EFFECT OF CHARGED-AMINO ACID OSMOLYTES ON PROTEIN STABILITY: SPECTROSCOPIC, VOLUMETRIC, AND COMPUTATIONAL STUDIES

A Thesis Submitted
for the Degree of

Doctor of Philosophy

by

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October 2018

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STATEMENT

I, Bramhini A, hereby declare that the work presented in this thesis, entitled as **"Effect of Charged-Amino acid Osmolytes on Protein Stability: Spectroscopic, Volumetric, and Computational Studies"** has been carried out by me under the supervision of *Dr. N. Prakash Prabhu* at Department of Biotechnology and Bioinformatics. To the best of my knowledge, this work has not been submitted for the award of any degree or diploma at any other university or institution. A report on plagiarism statistics from the University Librarian is enclosed.

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CERTIFICATE

This is to certify that the thesis entitled **"Effect of Charged-Amino acid Osmolytes on Protein Stability: Spectroscopic, Volumetric, and Computational Studies"** submitted by *Ms. Bramhini, A* bearing registration number 10LTPM04 in partial fulfilment of the requirements for award of Doctor of Philosophy in the Department of Biotechnology and Bioinformatics, School of Life Sciences is a bonafide work carried out by her under my supervision and guidance.

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1. Glutamate Induced Thermal Equilibrium Intermediate and Counteracting Effect on Chemical Denaturation of Proteins. Anumalla, B.; Prabhu, N. P., *J Phys Chem B* 2018, *122* (3), 1132-1144

B. Presented in the following conferences:

1. As a poster in "Innovations in Pharma and Biopharma Industry: Challenges and Opportunities for Academy and Industry (ICIPBI-2017)" organized at UoH-Dec 2017. (International)

2. As a poster in "BioQuest", a science conference organized at UoH-oct 2016. (National)
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1	BT505	Research Methodology	4	Pass
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3	BT506	Scientific Writing	1	Pass
5	BT459	Journal Club	1	Pass
6	BT571	Model Genetic Systems	2	Pass
7	BT572	Vaccine Technology	2	Pass

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In ever living memory of my friend

Vidyavathi Bai Shinde

Your selflessness will always be remembered girl

Acknowledgements

Although I am indeed the sole author of this thesis, I am by no means the sole contributor! So many people have contributed to my thesis, my education, and to my life and it is now my opportunity to thank them.

The first person who deserves my gratitude is my supervisor ***Dr. N. Prakash Prabhu*** *for his continuous support, patience, motivation, enthusiasm, and reinvigorating ideas. His guidance helped me in all the time of research and writing of this thesis. He has been supportive since the day I began working in his lab and sown the seeds of knowledge not only on research but also on different aspects of life. I feel incredibly privileged to have him as my supervisor.*

Besides my advisor, I would like to thank my thesis committee: Prof. J.S.S. Prakash and Dr. Sunanda Bhattacharyya for their encouragement and insightful comments. I would like to thank the present and former heads of Department of Biotechnology and Bioinformatics and also present and former deans of School of Life sciences for allowing me to use the departmental/School facilities. I am thankful to all the departmental and school non-teaching staffs for their help in doing all the official works in the department/school throughout this period of work.

I would like to thank University for providing me fellowship and also Department of Science & Technology (DST-India), CSIR for funding support to the lab.

I thank all my present and past fellow labmates for the discussions and for all the fun we have had in the past few years- Dr. Kiran (anna), Neshat (bahiyya), Tejaswi, Sanjay and also the Chelas needs a special mention-Pratibha, Shubhi and Archi will be remembered for your gatherings at my cubicle. Also I thank my former lab members-Chaitanya, Suraj, Hyma, Samshul, Deepak, Krishnakanth, Manikyaprabhu, Swathi, Dhanyasree, Sharath, Abhilash, Sreevidya,, Haider, Dipasree, Akanksha, Nishu, and present lab members-Sowmya, Ranjan and Mouli for making my stay stress free.

I am indebted to Sreevidya for introducing me to simulations and along with her the suggestions and timely assistance provided by Dr. Neshatul Haque and Sharath in analysis needs a special mention.

My heartfelt thanks to my friends Dr. Prashant Kumar, Dr. Deepak Babu, and Dr. Amit Ranjan for their seamless support throughout my study tenure and making my stay pleasant and memorable in the campus.

My special friends to thank Viddu and Spandana, I am extremely blessed to have you both but a major missing keeps happening. Those crazy memories are imperishable.

A special group "Family" is not mentioned yet, because they deserve their own part: the eternal love of all my family members is the anchor that holds me hard during the rough waters.

Foremost, I praise the enormous amount of struggle and pain taken by my parents throughout my childhood to see me successful. Mom-Dad, it's all your effort and faith that has inspired me to hold tight to my position no matter how complicated it gets. It's all your respect for education has, in some unconscious way, shaped my values and made me the person that I am today. It is because of your upbringing that I am at this stage and I promise I grow up to be the best I can be.

I am indebted to my mother-in-law, through good times and bad, her kindness and extensive support have been ever-present in this important duration of life. Without her willingness to be our primary caregiver all the time round the clock and especially her inexhaustible love for my son, this dissertation would have taken even longer to complete.

I am indebted to my husband for providing me with unwavering support and continuous encouragement throughout my years of study. I find it difficult to express my appreciation because it is so boundless. This accomplishment would not have been possible without you my R-coder. Success is in my stride, because I have a hubby like you by my side. I am grateful to my husband not just because he has given up so much to make my career a priority in our lives, but because he has seen me through the ups and downs of our journey together so far.

A special gratitude to my peddamma along with loving memories of peddananna. I am blessed for being the youngest of you all guys: sister and brother-in-law: Anitha-Sridhar; brothers and sister-in-laws: Mayanna-Keerthana, Kittanna-Shalini, and Manoj-Shwetha. Thank you all for being showered with all your love and affection. Yay! Here goes all the next gen kiddo's: Bubloo, Nani, Chinku, Bunny and the leading girl baby after me - Chutki needs a special mention for making me feel hopping with joy at times. The all-time query of my elder brother when he rings me up "When you will be done with your Ph.D?" rings in my ears as soon as he rings me up.

Last but not least, It's unworthy if I don't mention heartfelt sorry to my little hero for being away from him for so long hours in pursuit of degree. My heart goes walking around my tiny tot "Chinnu Papa", the bundle of joy with a big heart making me better chaperoned than before by letting me grow along with him. Being Chinnu mamma is my greatest blessing.

All that I cherish today is the grace of God. I thank the Almighty for granting me the strength, wisdom, knowledge and showering his blessings.

.... Bramhini, A.

ABBREVIATIONS

RNase A	-	Bovine pancreatic ribonuclease A
α-LA	-	Bovine alpha-lactalbumin
Arg	-	Arginine
Lys	-	Lysine
Asp	-	Aspartate
Glu	-	Glutamate
Gdm	-	Guanidinium chloride
NaCl	-	Sodium chloride
T_m	-	Transition midpoint temperature
ΔH_m	-	Enthalpy change at transition midpoint temperature
ΔC_p	-	Heat capacity change
ΔS_m	-	Entropy change at transition midpoint temperature
T	-	Temperature
CD	-	Circular dichroism
$K_{S\text{-}V}$	-	Stern-Volmer constant
V^o	-	Partial molar volume
K_s	-	Apparent molar adiabatic compressibility
ΔG_{unf}	-	Free energies of unfolding
C_m	-	Unfolding transition midpoint
m_g	-	Slope of the chemical denaturation transition
$\Delta\Delta G_T$	-	Change in unfolding free energy
stAAs	-	Stabilizing amino acids
MD	-	Molecular dynamic
RMSD	-	Root mean square deviation
SASA	-	Solvent accessible surface area
RDFs	-	Radial distribution functions
H-bonds	-	Hydrogen bonds
χ_hyd	-	Hydration fraction
n_w	-	Number of water molecules
n_AA	-	Number of amino acid molecules
r	-	Distance
N_w	-	Total number of water molecules
N_AA	-	Total number of amino acid molecules
$f_{P\text{-}W}$	-	Fraction of protein-water hydrogen bonds
$f_{P\text{-}AA}$	-	Fraction of protein-amino acid hydrogen bonds
$f_{P\text{-}Gdm}$	-	Fraction of protein-Gdm hydrogen bonds
BB	-	Backbone
SC	-	Side chain

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Chapter 1

Introduction

***1.1. Introduction*:**

Living organisms rely upon the proteins for complex and webbed cellular processes to make their survival. For these processes, a proper folding of the nascent polypeptide chain is essential. The spontaneous folding of a nascent polypeptide chain into a spatial three-dimensional native conformation has left the researchers in puzzle during the 1950s. However, the mystery of protein folding was slightly unfolded by the trailblazing contributions of Levinthal and Anfinsen. The puzzle so-called "Levinthal's paradox" states 'How the same native stable conformation of a protein is attained from a broad conformational ensemble of unordered structures although it seems to be stochastic?'.[1] Pioneering works of Anfinsen demonstrated that protein has to reach the global energy minimum by crossing all the possible local minima barriers to attain the most stable thermodynamic conformation, and also that the amino acid sequence has the crucial information required to drive the folding process.[2]

Protein folding is a complex process controlled by charge-charge interactions, hydrophobic interactions, and intra- and inter-molecular hydrogen bonding. An understanding of the forces responsible for the stability of native protein conformation is of great importance. Earlier works of Kauzmann,[3] and Dill[4] have shown hydrophobic, hydrogen bonding, and configurational entropy as major forces responsible for the stability of proteins. The balance between all these forces allows the protein to achieve a stable native conformation. Any disturbances in the balance could lead to the loss of its stable native conformation, thereby resulting in a functional loss.

It is a well-known fact that all the living systems share a basic feature of crowded internal cellular environment.[5-7] Apart from water, the interior of cells is majorly composed of several other macromolecules (approximately 40%) which have a significant influence on various biochemical pathways, thus, making the cells' internal environment crowded.[8] When a synthesized polypeptide chain undergoes folding it may form some unstable intermediate states which have the high propensity to aggregate in such a crowded environment.[9-10] In addition to this, the survival of organisms is a confronting task in stress conditions. Organisms which cannot adapt to any environmental stress will be at a risk. In the process of evolution, organisms when exposed to any stress conditions, such as extremes of temperatures, pH, salinity, and the presence of chaotropes, adapt to accumulate small organic molecules called osmolytes.[11-13] These small molecules are otherwise known as "*chemical* *chaperones*". [14] Osmolytes are known to play an essential role in stabilizing the proteins and folding of aberrant proteins *in vitro* as well as *in vivo*.[14-15] However, few instances of destabilizing osmolytes also have been identified.[16] Osmolytes are ubiquitous; their presence *in vivo* is detected all the way from halophiles to mammals.[17-18] Organisms are known to have many different osmolytes at a time and a specific osmolyte's activity is triggered as a response to a specific stress.

In general, osmolytes are classified into different categories based on two criteria: i) chemical structure, and ii) their effect on the functional activity of proteins as mentioned in Tables 1.1 & 1.2.[11] Compatible osmolytes are named so as they do not have any deleterious effects on the cells and its macromolecules.[19] Non-compatible osmolytes have negative effects on the macromolecules in the cells and may disrupt the functional activity of the proteins. Counteracting osmolytes are the ones which are evolved in response to the destabilizing effects of non-compatible osmolytes to counteract them.[20]

Table 1.1 Chemical classification of osmolytes

Class of osmolytes	**Examples**
Polyols and sugars	Sorbitol, Ethylene glycol, Glycerol, Myoinositol, and Mannitol. Trehalose, Glucose, Mannose, Sucrose, Raffinose, and Xylose
Amino acids and derivatives	Proline, Glycine, Alanine, Leucine, Isoleucine, Serine, Arginine, Lysine, Aspartic acid, Glutamate, and Phenylalanine. Taurine, Ectoine, β-alanine, and hypotaurine
Methylammonium compounds	Trimethylamine N-oxide(TMAO), Glycine betaine, Sarcosine, Glycerophosphocholine (GPC), and L-carnitine

Table 1.2. Functional classification of osmolytes

Class of osmolytes	**Examples**
Compatible	Sorbitol, Ethylene glycol, Glycerol, Trehalose, Proline, Glutamate, Taurine, and Ectoine
Non-compatible	Urea, Arginine, Histidine, and Lysine
Counteracting	TMAO, Betaine, and GPC (counteracts urea)

1.2. pH-dependent action of osmolytes:

Earlier studies show that the effect of polyols and sugars varies with pH and their effect is more pronounced at lower pH.[21] Polyols have varying effects on native and denatured states of the proteins.[22-23] Similarly, amino acids are expected to have pH-dependent stabilizing or destabilizing effect on proteins, but few studies have demonstrated that amino acids compatibility is independent of pH.[24-25] Methylamines act as potent stabilizers at neutral pH, while at lower pH they act as destabilizing agents.[26] For instance, TMAO, a zwitterionic osmolyte, acts as a potent stabilizer when its pH is greater than its pKa and at pH less than pKa (<5.0) it behaves as a denaturant.[27] Similarly, glycine betaine even exhibits a pH-dependent osmoprotectant activity. It serves as an excipient stabilizer for lysozyme and α-LA at a pH range of 6.0-8.0 whereas at lower pHs, it neither shows pronouncing stabilizing effects nor exhibits destabilizing effect. However, it is a potent stabilizer at all pHs for RNase A.[26, 28] Therefore, each osmolyte may be unique in responding to the stress conditions at varying pHs.

1.3. Natural selection of organic osmolytes over inorganic salts:

Nature’s selection of organic molecules as osmolytes over inorganic ions in cell volume regulation is an astonishing fact in spite of high economy of generation and utilization. For instance, photosynthetic cells utilize organic osmolytes at approximately 10 times higher metabolic cost to that of inorganic osmolytes.[29] Experiments have shown that the fluctuations in the levels of inorganic ions during the hypertonic conditions in the systems affect several biochemical processes. The hypertonic condition is associated with increased concentrations of inorganic solutes and several macromolecules along with the efflux of water from the cell's interior leading to cell shrinkage. Therefore, organic solutes with a limited permeability are preferred compared to inorganic salts.[7, 30-31] To combat from such adverse conditions, regulatory volume increase (RVI) is enacted by the accumulation of organic solutes by the cells to bring back to the isotonic cellular environment.[11, 32] Studies have shown that high salt concentrations in the cells, apart from changes in cell volume and ionic strength, induce DNA double-stranded breaks, and arrest the cell-cycle by up-regulating the p53 levels in the cells.[33] Similarly, in order to tolerate the stress conditions, halophiles accumulate inorganic salt K^+ even up to 5-7 M of concentrations. However, these high K^+ ions have to be counter-balanced by the accumulation of some negatively charged amino acid osmolytes such as glutamate to maintain the required ionic strength of the physiological systems.[34-35]

1.4. Proposed mechanisms on the osmolyte-induced protein stabilization:

Nearly five decades have been dedicated to understand the role of osmolyte molecules on protein stability, structure, and folding. Different theories have been proposed by the researchers on how these osmolytes confer stabilization to the proteins.[18, 36-39] However, no universal hypothesis has been established so far to explain the mechanism of action of osmolytes. The compatible osmolytes are known to shift the protein unfolding equilibrium N ↔ U toward left (Native) thereby stabilizing the proteins whereas the non-compatible osmolytes shift the equilibrium toward right (unfolded state).

1.4.1. Theory of preferential exclusion or interaction:

Timasheff and co-workers proposed this theory based on the differential effects of stabilizing and destabilizing osmolytes on the protein structure and stability. It states that the compatible osmolytes are preferentially excluded from the protein's surface. This exclusion is more from the unfolded state compared to the native state which drives the equilibrium toward the native state, thus stabilizing the protein.[40] This hypothesis corroborates with that of preferential exclusion of stabilizing Hofmeister ions. Further investigations have shown that the exclusion may be only from the denatured state and even a negative interaction coefficient of osmolyte with the protein also infers the same. The osmolytes that preferentially bind to the proteins are known to destabilize the proteins.[41] On the addition of any osmolyte to the protein solution, both interaction and exclusion of the osmolyte are observed despite its net interactive or exclusion property.[42] However, the net outcome of the balance between the preferential interaction and exclusion of osmolytes from the protein's vicinity determines their destabilizing or stabilizing property.[43-44]

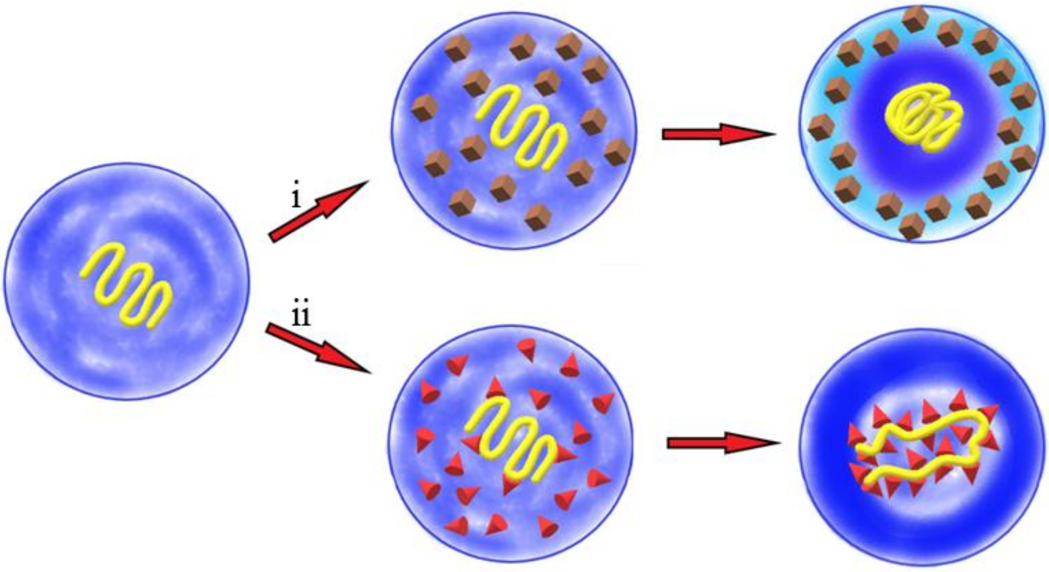

Figure.1.1. A schematic representation of preferential interaction or exclusion of osmolytes with protein. (i) Preferential exclusion of compatible osmolyte (brown cubes) from the protein's (yellow ribbon) surface leading to compaction. (ii) Preferential interaction of non-compatible osmolyte (red triangles) with the protein (yellow ribbon) resulting in denaturation. The surrounding water molecules are represented in blue where the intensity of the color is directly proportional to the density of water.

1.4.2. Transfer free energy measurements:

Free energy changes measured upon transfer of amino acid side chains and backbones from water to osmolyte solutions have shown that the osmolytes are being driven away from the protein's backbone due to their detrimental interaction which was termed as "osmophobic effect".[45-46] Further studies by Bolen and his co-workers on reduced and carboxamidated ribonuclease A (RCAM-RNase A) suggest that the major contribution of osmophobic interaction is from the protein's backbone while the side chains contribute a little.[45, 47] According to this theory, transfer of both the native and the unfolded protein to osmolyte solution would lead to the destabilization of protein. However, the unfolded state is more destabilized compared to the native state thereby inducing the stabilization to circumvent from the adverse effects of osmolytes.[48-49]

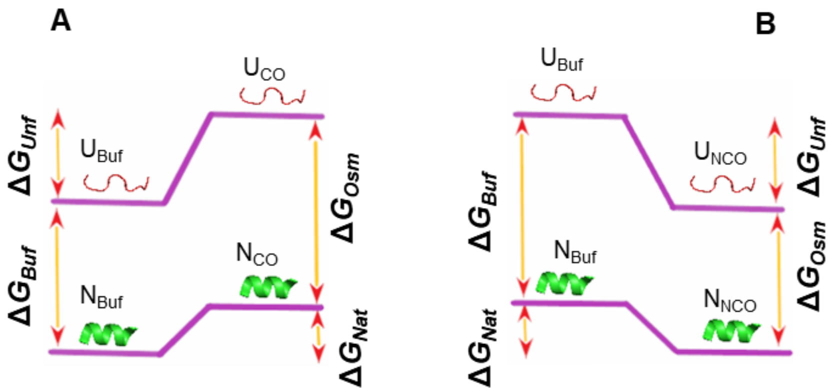

Figure.1.2. A schematic representation of free energy differences between native and unfolded states of a protein in buffer (**ΔG_{Buf}**) and in osmolyte (**ΔG_{Osm}**) solution. Panels A and B depict the changes in the presence of a compatible osmolyte (CO) and a non-compatible osmolyte (NCO), respectively. Adapted from ref 50.

Further evidences from molecular dynamic (MD) simulation studies help us to understand the osmolytes interaction with protein and their effect on water structure around the protein, and in the bulk at the molecular level. Earlier simulation studies with polyols and methylamines suggest that the exclusion of osmolytes from the protein's vicinity and reordering of the water structure are the major factors behind the osmolyte-induced stabilization. Unfavourable interactions of methylamines with the proteins along with the enhancement of solvent-solvent (water-water or water-urea) interactions are shown to stabilize proteins.[50-51] But polyols are found to disrupt the tetrahedral water structure by forming linear H-bonds with waters.[52] However, these effects could not be generalized to all the osmolytes and the proteins. For instance, trehalose, in spite of being a stabilizer, binds to the proteins.[53] Recent investigations with amino acids also support the above observations. The preferential exclusion of proline and the preferentially binding of arginine with the proteins making them stabilizing and destabilizing osmolytes, respectively.[54-55] All these observations from MD simulations reemphasize the hypothesis of Timasheff *et al.*, that it is the preferential exclusion or interaction of osmolyte with the protein that drives the stabilization or destabilization of proteins, respectively.

1.5. *Applications of osmolytes in health and biotechnology industries*:

1.5.1. Assist in protein trafficking and misfolding disorders:

Various clinical and *in vitro* investigations suggest that some of the human diseases are associated with the flawed folding of nascent polypeptide chains. This disease associated misfolded proteins may be functionally inert or have a transformed activity or may be mis-localized in the cells.[56-57] Phenylketonuria, dialysis-related amyloidosis, cystic fibrosis, amyotrophic lateral sclerosis, serpin deficiency disorders, hemolytic anemia, transmissible spongiform encephalitis, etc., are the various protein conformational diseases associated with the misfolding of different proteins.[58-59] Several studies have brought into the light the role of small molecules in correcting the erroneous conformations evolved due to misfolding and restoring the functional activity of the proteins.[14, 60-62] For instance, nephrogenic diabetes insipidus occurs due to point mutations associated with aquaporin-2. However, 1.0 M glycerol was found to correct the trafficking and misfolding of mutant aquaporin-2.[63] Cystic fibrosis is associated with the mutant delta F508 form of cystic fibrosis transmembrane conductance regulator protein. This was shown to be properly transported to the plasma membrane to execute its function upon supplementing with glycerol or TMAO.[60] Hence, understanding of the osmolyte-protein interactions could help in designing the osmolyte based therapies. Moreover, their usage as an additive by biotech industries in recombinant protein synthesis to prevent from misfolding, thus improving their production yield, has been promising.[64]

1.5.2. Role in prevention of aggregation and fibrillation of proteins:

At certain conditions, proteins tend to attain non-native conformations and polymerize to form more stable complexes called aggregates which may be toxic or biologically inactive. Aggregation can be either due to extrinsic (environmental changes) or intrinsic factors (structural changes). Some of the diseases associated with the deposition of aggregated or fibrillar proteins are Alzheimer’s, Parkinson’s disease, spinocerebellar ataxia, primary systemic amyloidosis, frontotemporal dementia, lysozyme systemic amyloidosis, Creutzfeldt-Jakob disease, Huntington’s disease, diabetes mellitus II, and insulin-related amyloid.[58, 65-67] Protein aggregation is a recurring challenge encountered by bio-pharmaceutical industries during the production of therapeutic proteins. Osmolytes are proven to be excellent excipient molecules to tackle the aggregation. However, not all the osmolytes suppress the aggregation while some of them are found to induce the aggregation. Moreover, the effect of osmolytes may not be the same across the proteins.[68] Therefore, understanding the self-aggregation of proteins and osmolyte-intervention mechanisms are essential for rationally designing the strategies to evade this problem.[69-70]

***1.5.3. Chemical chaperones do influence molecular chaperones*:**

Osmolytes are also known to regulate molecular chaperones in the cells. During the combined salt and heat stresses osmolytes are found to trigger the molecular chaperones (GroEL, DnaK and ClpB) assisted disaggregation and refolding of proteins.[71] When betaine, proline, glycerol, and trehalose were supplemented to *E. coli* cells to evaluate their effect on proteins under high saline and heat stress conditions, they were able to refold the malate dehydrogenase (except trehalose).[71] These osmolytes were able to protect the proteins against salt and heat stress by activating a single or multiple chaperones, thereby refolding the protein and finally inducing stabilization of refolded protein.[71] Similarly, K- glutamate and glycine betaine naturally accumulating in salt- stressed cells are shown to modulate the activity of chaperone ClpB. Also, they counter protect the chaperones from the inhibitory effects of other small molecules in the cells, thus, improving the efficiency of chaperone-assisted protein disaggregation and refolding.[72] In yeast Hungtinton's model, trehalose not only solubilizes the mutant aggregates, but also increases the cell viability by increasing the expression of Hsp104p by repairing the endocytosis machinery.[73-74] In addition to Hsps, Hsf1 activity is also strongly influenced by the presence of trehalose in the cells by meddling with the feedback regulation of Hsf1 by HSPs.[75] Apart from this, a combination of salt-induced osmolyte accumulation and benzyl alcohol-induced chaperone accumulation is shown to have efficient protein refolding capability than when they are used individually.[76]

***1.5.4. Osmolytes as cryo-protectants*:**

Cryopreservation has become an emerging technology in the fields of biomedical engineering, cell line development, drug screening, and conservation of biological species. Osmolytes have potent applications as cryoprotectant solutes when cells or organisms are to be stored or grown under cryo-temperatures. Employment of cryopreservation was started with the discovery of glycerol as a cryoprotectant solute for storing eukaryotic cells.[77] Cryoprotectants are basically categorized into permeable and non-permeable based on their penetration capacity through the cell membrane. However, the characteristic property of cryoprotectant is dependent on the conditions of the freezing temperature and the cell type.[78] Several alcohols, polyols, saccharides, disaccharides, and amino acids are used in protecting different species from freezing temperatures. However, amino acids are not as effective as sugars and polyols. Amongst all, trehalose and proline are the natural cryoprotective additives (CPA).[79-80] Despite having wider applications, the recovery of cells and functional activities in the presence of CPA's is not 100%, although better than the survival rates of cells in their absence.[79, 81] In addition to this, synergistic effect of CPA's is observed when two or more osmolytes mixtures areare used in cryoprotection.[82-83]

1.5.5. Excipients during lyophilization:

Lyophilization allows the proper storage and transportation of cells at room temperature. This may damage the cell membrane and decrease the cell viability during the process. Osmolytes, when added during the lyophilization procedure to the cells, are capable of keeping the membrane intact and in enhancing the cells viability as well.[84] For example, sucrose, dextran, and trehalose stabilize lysozyme protein when added as excipients during its long-term storage by water substitution mechanism.[85] Osmolytes even found an important role as excipients in vaccine development. Freeze-drying of vaccine in the presence of trehalose showed an improved stability of the vaccine at optimal temperatures and for longer durations.[86] Addition of amino acids at very low amounts to the sucrose-based protein formulations during lyophilisation enhanced the storage stability by 50%.[87] In addition, mixtures of amino acids also can be employed to improve the stability and recovery without any toxic effects on the proteins during lyophilisation as studied in the case of recombinant factor VIII.[88]

1.5.6. Osmoprotectant activity during cell-volume regulation:

Cellular milieu must preserve the hospitable environment for the proper physiological functioning of various macromolecules in the system. The activities of these macromolecules are affected by varying the composition of cellular water and also the type of solutes and their concentrations.[89] Although few osmolytes are selective for certain species, most of the osmolytes are conserved in phylogenetically diverse species. Therefore, the mechanisms involved to up- or down-regulate the levels of osmolytes in the cells might be common across different species.[90] These osmolyte molecules are considered to be evolved parallelly with the evolution of cellular systems to provide an apt intracellular environment for the biological systems. As discussed in the earlier section, during hyperosmotic or hypoosmotic stress conditions, the type and levels of osmolyte molecules vary to favor the condition either by intake from media, by de novo synthesis or by efflux of these molecules into the external environment.[91]

This paradoxical behavior of the osmolytes makes it imperative to investigate the impact of each osmolyte on distinct proteins. Therefore, a clear understanding of the multifaceted roles of these organic solutes might emerge and could help us to find their feasible applications. Extensive studies on the effects of polyols, sugars, hydrophobic amino acids, and methylamines on different proteins have been carried out for last five decades[11, 17, 22, 24, 92-95], however, not many investigations are carried out on the charged amino acids to understand their effects on protein stability. Based on the above groundwork, this thesis examines the effect of four charged amino acids glutamate (Glu), aspartate (Asp), arginine (Arg), and lysine (Lys) on the thermodynamic stability of two model proteins, bovine pancreatic ribonuclease A (RNase A) which has net positive charge and bovine alpha-lactalbumin(α-LA) which has net negative charge at pH 7. RNase A, an endonuclease by function, is a single domain protein consisting of 124 amino acid residues with a molecular mass of 13.7 kDa.[96] α-LA is a Ca^{2+} binding milk protein with a molecular weight of 14.2 kDa.[97] It is a regulatory protein involved in the lactose biosynthesis.[98] Recent findings show that α-LA has a significant role in the apoptosis of tumor cells as well.[99] We evaluate here whether the amino acids induced effects varies with the proteins surface charge using spectroscopic and volumetric studies. The experimental results are supplemented with molecular dynamic simulation studies to understand the molecular level interactions. In addition, the counteracting effect of compatible amino acids against the non-compatible amino acids is also examined.

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Chapter 2

Glutamate-Induced Thermal Equilibrium Intermediate and Counteracting Effect on Chemical Denaturation of Proteins

2.1. *Abstract*:

The present chapter examines the effect of a negatively charged amino acid osmolyte, glutamate (Glu) on the thermodynamic stability of two model proteins ribonuclease A (RNase A) and α-lactalbumin (α-LA) which have positive and negative surface charges at pH 7, respectively. These proteins follow two-state unfolding transitions during both heat and chemical induced denaturation processes. The addition of Glu stabilizes the proteins against temperature and induces an early equilibrium-intermediate during unfolding. The stability is found to be enthalpy-driven and the free energy of stabilization is more for α-LA compared to RNase A. The decrease in partial molar volume and the compressibility of both the proteins in the presence of Glu suggests that the proteins attain more compact state through surface hydration which could provide a stable conformation. Further, the intermediates could be completely destabilized by lower concentrations (~0.5 M) of guanidinium chloride and salt. Urea subverts the Glu-induced intermediate formed by α-LA whereas it only slightly destabilizes in the case of RNase A which has positive surface charge and could possess charge-charge interactions with Glu. This suggests that apart from the hydration, columbic interactions might also contribute to the stability of the intermediate. Guanidinium chloride-induced denaturation of RNase A and α-LA in the absence and in the presence of Glu at different temperatures were carried out. These results also show that Glu increases the stability of both the proteins; however, all the unfolding transitions followed two-state transitions during chemical denaturation. The extent of stability exerted by Glu is higher for RNase A at higher temperatures, whereas it provides more stability for α-LA at lower temperatures. Thus, the experiments indicate that Glu induces a thermal equilibrium intermediate and increases the thermodynamic stability of proteins irrespective of their surface charges. The extent of stability varies between the proteins in a temperature-dependent manner.

2.2. Introduction:

The conformational stability of proteins in the presence of any cosolvent can be assessed by employing the conventional techniques such as denaturation of proteins by gradually increasing the temperature or denaturant concentration.[1] Although several small globular proteins exhibit two-state (re)unfolding pathway, some of them might deviate by forming one or more intermediates during their (re)unfolding.[2-6] Structual and energtic studies of these intermediates can provide us deeper insights into the folding mechansim and help to remodel the classical energy landscape.[7] However, most of the times, these intermediates are spectroscopically indistinguishable from their extreme states, native and unfolded, making their detection a confronting task.

Osmolytes are known to assist the refolding of several misfolded/aggregated proteins either by enhancing the folding rate or by inducing intermediates or by binding to the proteins.[8-9] Earlier reports suggest that osmolytes influence the dynamics by reducing the heterogeneity of conformations available in the solution instead of inducing the significant structural changes either in the native or unfolded proteins.[10-11] Therefore, osmolytes are expected to show their impact on the transition region or intermediates involved.[12] However, a very few studies have identified their ability to induce intermediate during the folding pathway of proteins.[13] For instance, TMAO and sarcosine induces an early intermediate during the folding of Barstar protein; however, the structures induced by these osmolytes are not similar as the formation of this early intermediate itself is a multi-step process and possess many forms in the ensemble.[14] Moderate concentrations of TMAO also induces an intermediate during the folding of α-synuclein and further lead to fibrillation whereas higher concentrations induce oligomerization.[15] Addition of sugars to acid-denatured cytochrome *c* induces a compact stable intermediate by enhancing the steric repulsions among the solution components which is similar to that of A-state. [16]

Among the different chemical classes of osmolytes, amino acids and their derivatives are the dominant solutes in phylogenetically diverse organisms such as halophiles, halophytes, marine invertebrates, and hagfishes.[17-18] An earlier study conducted in *E.Coli* has shown that L-amino acids are better osmo-protectants against environmental stress like cold, heat, and salt, while their D-isomers show an inhibitory effect. However, either of these effects can be reversed by varying the temperature.[19] Amino acids are also shown to have a vital role in regulating the osmotic stress in brain cells and kidney tissues.[20] Many *in vitro* studies show that amino acids and their derivatives can inhibit protein aggregation and fibril formation. In order to prevent the aggregation during the refolding of proteins, charged and some of the hydrophobic amino acids are commonly used in protein purification.[21-23] Among them, arginine is considered to be more effective for many of the proteins with minimal effect on their thermodynamic stability.[23-27] Further, amino acid derivatives such as ectoine, betaine, and citrulline could inhibit insulin amyloid formation by eliminating the hydrophobic interactions among the protein monomers and by interacting with the hydrophilic regions on the protein surface.[28]Amidated derivatives of Ala, Arg, Asn, Met, and Val slow down the heat-induced inactivation and aggregation of proteins.[29-31]

In spite of several studies on polyols[32,33,34] and amino acid classes[33,35,36] of osmolytes, the effect of negatively charged amino acid glutamate (Glu) on protein stability is less explored.[37-38] Nonetheless, the osmo-protectant property of Glu in different organisms has been known for a quite long time.[39-41] For instance, glutamine protects the mouse zygotes from high salt concentrations during the early developmental stages.[42-43]Also, it helps in self-association of tubulin to form microtubules.[40] A study on glutaminyl-tRNA synthetase and the S228N mutant of λ-repressor shows that glutamate enhances the stability of these proteins against urea denaturation presumably by destabilizing their unfolded states.[38] However, its exact action on the thermodynamic stability of proteins against temperature- and guanidinium chloride-induced unfolding is yet to be investigated. In this chapter, we report the effect of glutamate on two globular proteins, ribonuclease A (RNase A) and α-lactalbumin (α-LA). Both the proteins show two-state unfolding transitions against chemical and thermal denaturation in the absence of any added cosolvents.[44-46] Further, they have different surface charges and pIs (RNase A, 9.6; α-LA, 4.8). Hence, the effect of a charged amino acid osmolyte, Glu, on these proteins could explain the mechanism of action between the protein and Glu. The results show that Glu stabilizes the proteins against temperature and chemical-induced denaturation. Interestingly, in higher concentrations of Glu, both the proteins follow three-state thermal unfolding transitions with spectroscopically detectable intermediate. The extent of stabilization varies between the proteins and is temperature-dependent. The spectral and volumetric results suggest that Glu might operate through preferential hydration mechanism in stabilizing the proteins.

2.3. Experimental methods:

2.3.1. Materials:

Guanidinium chloride (Gdm), L-glutamate (Glu), sodium phosphate dibasic, sodium phosphate monobasic, urea, and sodium chloride (NaCl) were purchased from SRL. Bovine pancreatic ribonuclease A (RNase A) and bovine α –lactalbumin (α-LA) were from Sigma. All the chemicals were used without any further purification.

2.3.2. Thermal denaturation studies:

Thermal denaturation studies of the proteins in 20 mM phosphate buffer at pH 7.0 were carried out in the absence and the presence of different concentrations of Glu. Change in the absorbance with increasing temperature was followed at 285 nm for both the proteins. All the samples were heated from 20 to 90 °C with a scan rate of 1 °C per min using a Peltier attached with the spectrophotometer (Cary 100, Agilent). The concentrations of the proteins were ~15 µM. The thermal denaturation curves showing sigmoidal two-state transitions were analyzed using the following equation: [47]

$$Y = \frac{(y_f+m_fT) + (y_u+m_uT)e^{\Delta Hm\left(\frac{T}{Tm}-1\right)+\frac{\Delta Cp\left(Tm-T+\left(Tln\frac{T}{Tm}\right)\right)}{RT}}}{1 + e^{\Delta Hm\left(\frac{T}{Tm}-1\right)+\frac{\Delta Cp\left(Tm-T+\left(Tln\frac{T}{Tm}\right)\right)}{RT}}} \qquad (1)$$

The denaturation curves with three-state transitions were analyzed using the following equation with an assumption that the change in ΔC_p with varying temperature is insignificant ($d\Delta C_p/dT \approx 0$),[48]

$$Y = \frac{(y_f+m_fT) + (y_i+m_iT)e^{\left(-\frac{\Delta G_1}{RT}\right)} + (y_u+m_uT)e^{\left(-\frac{\Delta G_1+\Delta G_2}{RT}\right)}}{1 + e^{\left(-\frac{\Delta G_1}{RT}\right)} + (y_u+m_uT)e^{\left(-\frac{\Delta G_1+\Delta G_2}{RT}\right)}} \qquad (2)$$

where

$$\Delta G_1 = \Delta H_{m1}\left(1 - \frac{T}{T_{m1}}\right) + \Delta C_{p1}\left(T - T_{m1} - \ ln\frac{T}{T_{m1}}\right)$$

$$\Delta G_2 = \Delta H_{m2}\left(1 - \frac{T}{T_{m2}}\right) + \Delta C_{p2}\left(T - T_{m2} - \ ln\frac{T}{T_{m2}}\right)$$

In the equations, Y is the normalized values of absorbance; y_f, y_i and y_u correspond to the absorbance values of stationary phases between the transitions, and m_f, m_i and m_u are their slopes, respectively.T_{m1} and T_{m2} are the transition midpoint temperatures, ΔH_{m1} and ΔH_{m2} are the enthalpy changes at the midpoint transition temperatures, and ΔC_{p1} and ΔC_{p2} are the heat capacity changes associated with the transition phases in the protein unfolding process. T is temperature and R is the gas constant.

2.3.3. Chemical denaturation studies:

Gdm-induced unfolding of the proteins was followed by intrinsic fluorescence changes of the proteins using Fluoromax-3 (Horiba) spectrofluorometer equipped with a Peltier for temperature control. The emission spectra of the proteins were recorded from 290 to 310 nm and from 300 to 380 nm for RNase A and α-LA, respectively after exciting the proteins at 280 nm. The spectra were obtained in the absence and the presence of 0.25, 0.5, and 1.0 M of Glu and at five different temperatures, 293, 298, 303, 308, and 313 K. In all the experiments, the concentrations of the proteins were 15 µM in 20 mM phosphate buffer at pH 7.0. All the denaturation curves were analyzed using the following equation with an assumption of two-state transition:[49]

$$Y = \frac{(y_f + m_f[Gdm]) + (y_u + m_u[Gdm])e^{\left(\frac{-\Delta G_{unf} + m_g[Gdm]}{RT}\right)}}{1 + e^{\left(\frac{-\Delta G_{unf} + m_g[Gdm]}{RT}\right)}} \qquad (3)$$

where Y is the normalized spectroscopic signal; y_f and y_u correspond to the intercepts, and m_f and m_u correspond to the slopes of pre- and post-unfolding baselines, respectively. ΔG_{unf} is the free energy of unfolding and m_g is the slope of the transition region.

2.3.4. Circular dichroism spectra:

Tertiary structural changes of the proteins in the presence of Glu were analyzed at near-UV region of circular dichroism (CD) in Jasco J-1500 spectropolarimeter using the protein concentration of ~15 µM. At far-UV region of CD, the detector voltage increased tremendously in the presence of Glu which hindered the measurement of ellipticity changes in the proteins. This could be due to the interference of Glu's absorption in this region.[50]

2.3.5. Fluorescence quenching:

The intrinsic fluorescence of the proteins was measured in the presence of increasing concentrations of a neutral quencher, acrylamide. The quenching effect was evaluated by Stern-Volmer constant ($K_{S\text{-}V}$) using the following equation,

$$\frac{F_0}{F} = 1 + K_{S-V}\,[acrylamide] \qquad (4)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. $K_{S\text{-}V}$ is the quenching constant.

2.3.6. Volumetric measurements:

Partial molar properties were measured from density and acoustic measurements using DSA-5000M (Anton-Paar) instrument with an in-built temperature controller. The partial molar volume (V^o) was calculated from the following relation:[51]

$$V^o = {}^{M_w}\!/_{\rho_o} - \left({}^{\rho - \rho_o}\!/_{\rho_o C}\right) \qquad (5)$$

where ρ and ρ_o are the densities of the solution and solvent, respectively. M_w is the molecular weight of the protein, and C is the molar concentration. The apparent molar adiabatic compressibility (K_s) was derived from the molar sound velocity $[U] = (u\text{-}u_o)/u_oC$, where u and u_o are the sound velocities of the solution and solvent, respectively using the following equation[51]

$$K_s = \beta_o(2V^o - 2[U] - {}^{M_w}\!/_{\rho_o}) \qquad (6)$$

Where β_o is coefficient of adiabatic compressibility of the solvent which is equivalent to $1/(\rho_o.u_o^2)$.

2.3.7. Electrostatic potential map:

Electrostatic surface potential maps of the proteins were generated in Pymol using Adaptive Poisson Boltzmann solver (ABPS) to calculate the electrostatic properties.[52-54] Input PQR files were prepared from PDB2PQR web server using the crystal structures of RNase A (PDB id: 7RSA) and α-LA (PDB id: 1HFX) obtained from the protein data bank. PROPKA was used to assign the protonation states of the proteins at pH 7.0.[55]

2.4. Results:

2.4.1. Thermal denaturation studies:

The effect of Glu on the stability of two model proteins, RNase A, and α-LA was studied by following the thermal denaturation of the proteins using change in their absorbance values. The denaturation followed a two-state transition, native ↔ unfolded, in the absence of Glu (Figure 2.1). The data were fitted to equation 1 and the T_m values were calculated to be 338.2 and 329.3 K for RNase A and α-LA, respectively. In the presence of Glu, the thermal transitions of both the proteins were shifted towards higher temperature suggesting stabilization of the proteins against temperature.

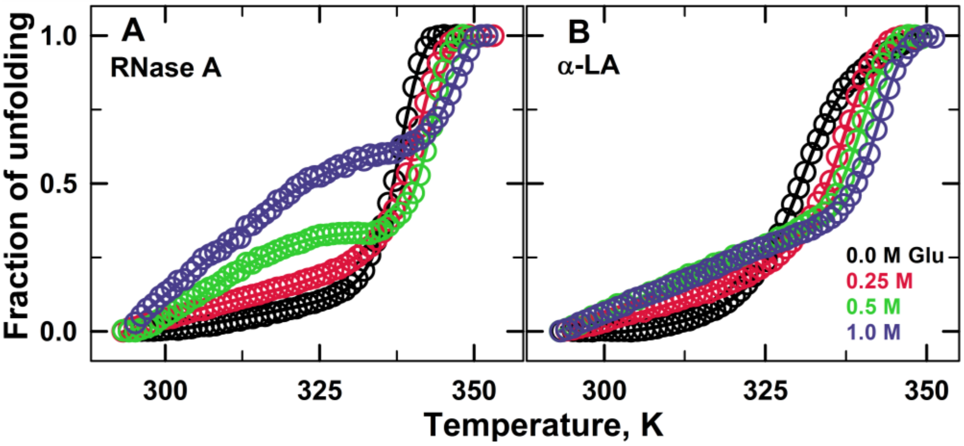

Figure 2.1. Thermal denaturation profiles of RNase A (A) and α-LA (B) in the absence (black) and the presence of 0.25 M (red), 0.5 M (green) and 1.0 M (blue) Glu. Solid lines represent the data fitted using equation 1 or 2. The thermodynamic parameters obtained are presented in Table 2.1.

However, the sigmoidal feature of two-state transition was lost when Glu concentration was above 0.25 M. This could be attributed to the formation of an intermediate during the unfolding reaction. Therefore, the denaturation curves obtained in the presence of 0.5 and 1.0 M of Glu were analyzed with a three-state assumption, native ↔ intermediate ↔ unfolded (N ↔ I ↔ U), for both the proteins using equation 2. This improved the fit with minimum residual values compared to the two-state assumption (Figure 2.2). All the parameters obtained from the curve fits are presented in Table 2.1.

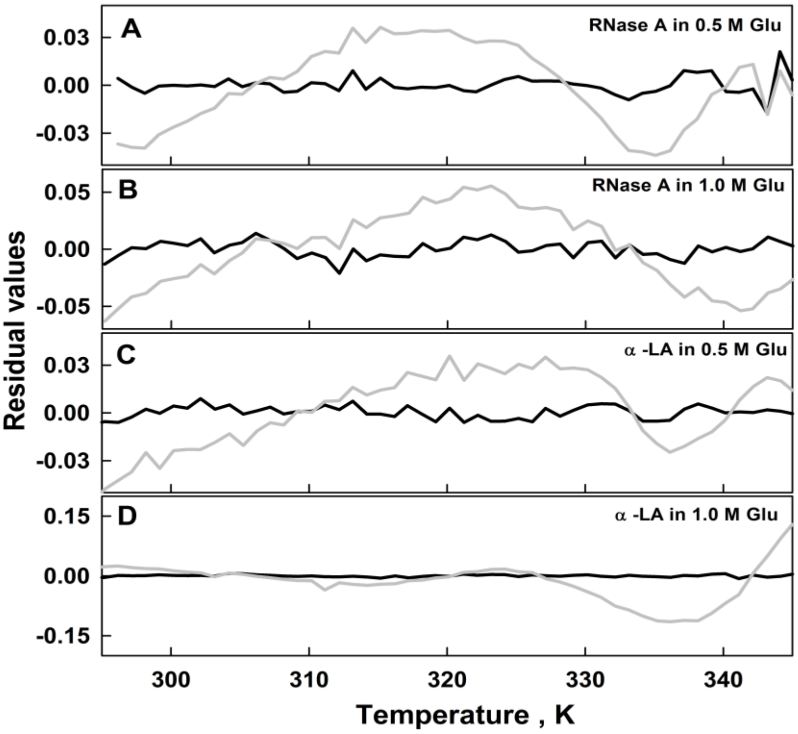

Figure 2.2. The residual values obtained from fitting the thermal denaturation of RNase A (A and B) and α-LA (C and D) in higher concentrations of Glu by two-state (gray) and three-state (black) assumptions. The denaturation transitions are presented in Figure 2.1.

Table 2.1: Thermodynamic parameters obtained from thermal denaturation studies§

	No Glu†		**0.25 M Glu**†		**0.5 M Glu**‡		**1.0 M Glu**‡	
	T_m	ΔH	T_m	ΔH	T_m	ΔH	T_m	ΔH
				RNase A				
N ↔ I	--	--	--	--	320.0±0.2	20.6±2.0	319.7±0.2	20.0±2.0
N or I ↔ U	338.2±0.1	105.0±2.4	341.0±0.1	110.0±2.6	343.6±0.2	117.7±2.8	347.4±0.2	126.0±2.8
				α-LA				
N ↔ I	--	--	--	--	318.0±0.3	20.6±2.0	318.0±0.3	21.1±2.0
N or I ↔ U	329.3±0.1	56.3±1.1	337.4±0.2	77.0±2.1	340.0±0.4	98.0±2.1	342.9±0.4	99.1±2.1

$^{\S}T_m$ in kelvin and ΔH in kcal/mol units; †two-state and ‡three-state unfolding;

Also, the fractions of each species, native, intermediate and unfolded, with increasing temperature were calculated in varying concentrations of Glu for both the proteins (Figure 2.3). The highest populations of intermediates were found to be at 338(±1) K and 334(±1) K for RNase A and α-LA, respectively.

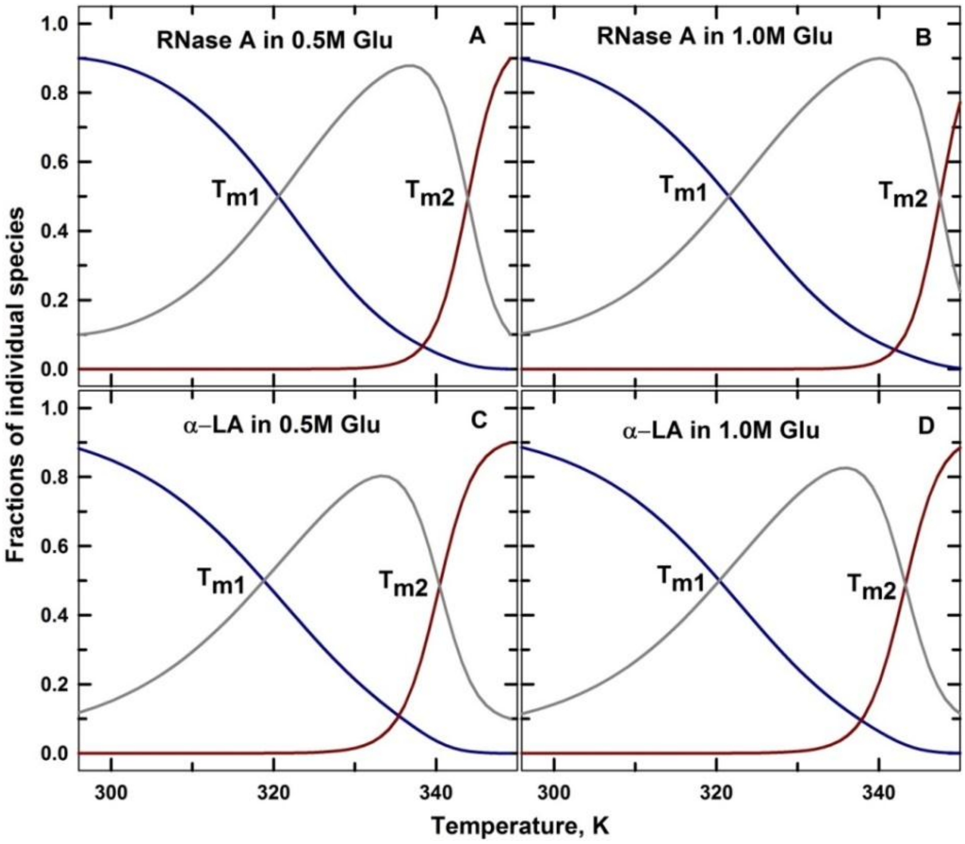

Figure 2.3. Fractions of native (blue), intermediate (gray), and unfolded (red) conformations at different temperatures during the thermal denaturation of RNase A (A and B) and α-LA (C and D) in the presence of 0.5 M or 1.0 M of Glu.

2.4.2. Perturbation of equilibrium intermediate:

For further characterization of the equilibrium intermediate induced by Glu, thermal denaturation studies were carried out in the presence of Gdm, an ionic denaturant; urea, a neutral denaturant; and sodium chloride, an ionic salt. The concentrations of denaturants were chosen such that the native structures of the proteins were not significantly altered. The secondary structure of RNase A was almost completely retained in 2 M Gdm or 3 M urea or 1 M salt, whereas α-LA was stable only at lower concentrations of the denaturants, i.e., 1 M Gdm or 1.5 M urea (Figure 2.4). It may be noted that the enthalpy of unfolding and the transition midpoint of α-LA was less compared to RNase A (Table 2.1).

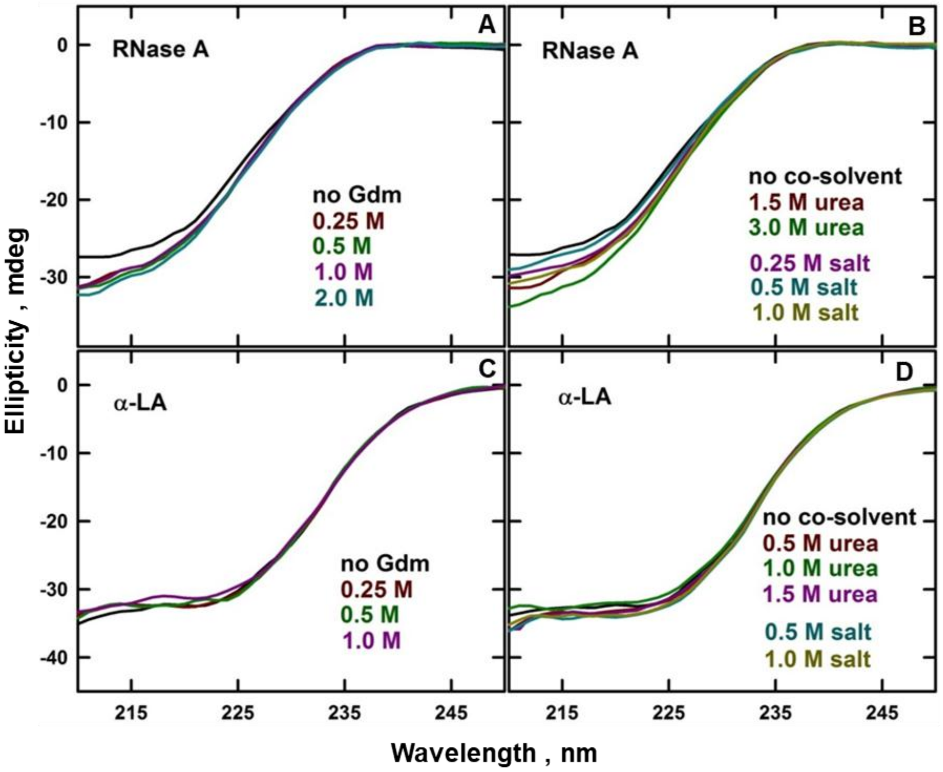

Figure 2.4. Secondary structural contents of RNase A and α -LA analyzed using far-UV circular dichroism spectra of the proteins in different co-solvents. CD spectra of RNase A in the presence of varying concentrations of Gdm (A), and urea & salt (B). CD spectra of α-LA in the presence of varying concentrations of Gdm (C), and urea & salt (D).

In the absence of Glu, an early denaturation of RNase A was observed in the presence of both Gdm and urea (Figures 2.5A and 2.5E). As an ionic denaturant, Gdm was more effective in destabilization of the protein than urea which is commonly observed in other proteins as well. [56-57] Only slight changes were observed on the thermal denaturation of RNase A in the presence of salt (Figure 2.5E). Addition of Gdm, urea or salt did not affect the two-state transition of the protein. In the presence of 0.25 M Glu as well, the effects of Gdm, urea, and salt on the protein were similar (Figures 2.5B and 2.5F). When Gdm was added in the presence of higher concentrations of Glu, the thermal denaturation showed two-state transition without any observable intermediate state even with the lowest concentration of Gdm employed, i.e., 0.5 M (Figures 2.5C and 2.5D). Though the addition of urea also could destabilize the intermediate formed in the presence of 0.5 M Glu (Figure 2.5G), a three-state transition was still observed in 1.0 M Glu even with the addition of 3 M of urea (Figure 2.5H). Addition of salt also destabilized the intermediate and shifted the unfolding to two-state transition (Figure 2.5G). The data were fitted to equation 1 (two-state) or 2 (three-state) and the parameters are presented in Figures 2.6 & 2.7 and in Table 2.2.

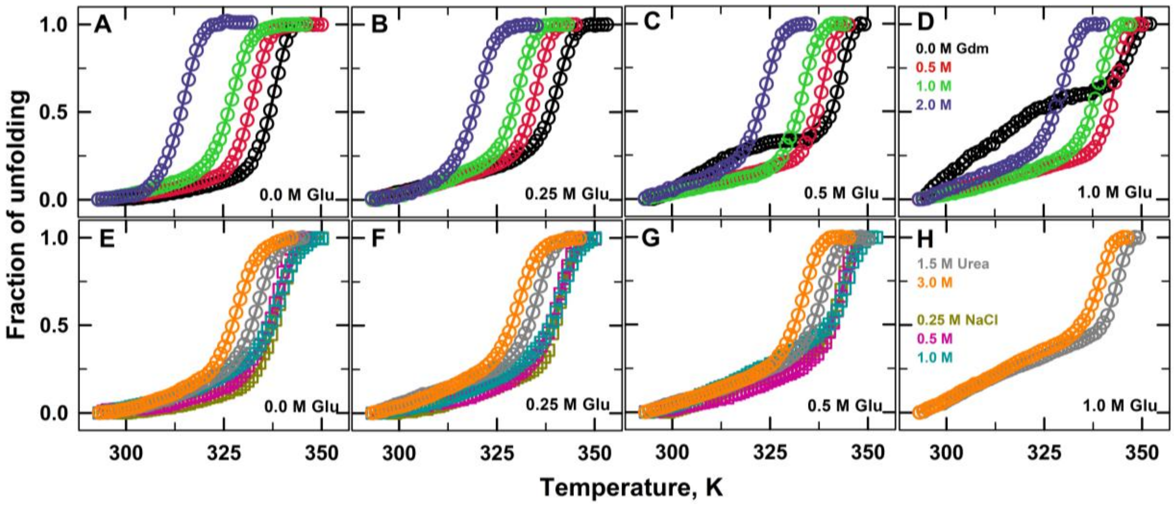

Figure 2.5. The effect of Gdm (upper panels), urea and salt (lower panels) on the thermal denaturation of RNase A in varying concentrations of Glu monitored using absorbance changes. The color codes represent the concentrations of cosolvents as mentioned in the figure labels. The solid lines represent the data fitted using equation 1 or 2. The thermodynamic parameters obtained are presented in Table 2.2.

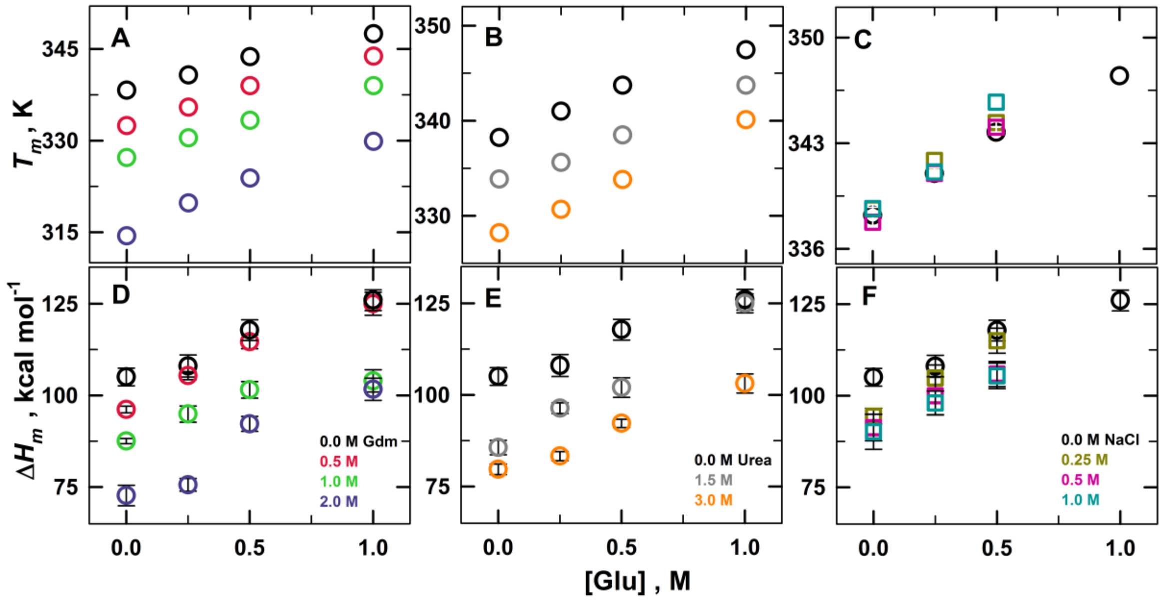

Figure 2.6. Changes in transition midpoint of thermal denaturation, T_m (upper panels) and enthalpy of unfolding, ΔH_m (lower panels) of RNase A in Glu upon the addition of varying concentrations of Gdm (A and D), urea (B and E), and salt (C and F). The color codes represent the concentrations of cosolvents as mentioned in the figure labels. In the case of three state transitions, the parameters of the major transition (I ↔ U) are plotted.

Table 2.2. Thermal denaturation of RNase A in denaturants and salt[§]

Cosolvent	No Glu		0.25 M Glu		0.5 M Glu		1.0 M Glu	
	T_m	ΔH	T_m	ΔH	T_m	ΔH	T_m	ΔH
Gdm								
0.5M	332.4±0.0	96.1±0.9	335.0±0.1	109.5±2.0	338.9±0.1	114.6±1.9	343.8±0.1	125±3.1
1.0M	327.2±0.0	87.5±0.6	330.4±0.1	94.8±2.2	333.3±0.1	101.5±2.2	338.9±0.2	104.0±3.0
2.0M	314.4±0.2	72.7±2.8	319.8±0.2	75.6±1.7	323.8±0.2	92.2±2.0	329.8±0.1	101.6±3.0
Urea								
1.5M	333.8±0.1	85.6±1.9	335.6±0.0	96.3±1.5	338.5±0.1	102.0±2.6	343.7±0.2	125.0±2.6
							(312.0±0.5)[†]	(18.0)[†]
3.0M	328.0±0.0	79.7±1.4	330.6±0.0	83.3±1.2	333.7±0.0	92.2±1.1	340.2±0.2	108.0±2.6
							(311.9±0.5)[†]	(15.0)[†]
Salt								
0.25 M	338.6±0.1	94.4±2.1	341.9±0.2	104.8±3.5	344.4±0.2	115.0±3.4	-	-
0.5 M	337.7±0.3	91.3±3.6	341±0.3	100.0±5.1	344.0±0.3	105.8±3.4	-	-
1.0 M	338.6±0.2	90.1±4.7	341.1±0.2	98.0±3.2	345.7±0.3	105.4±3.4	-	-

[§] T_m in kelvin and ΔH in kcal/mol units; [†] in three-state transitions, T_m and ΔH values of the first transition (N↔ I) are in parentheses;

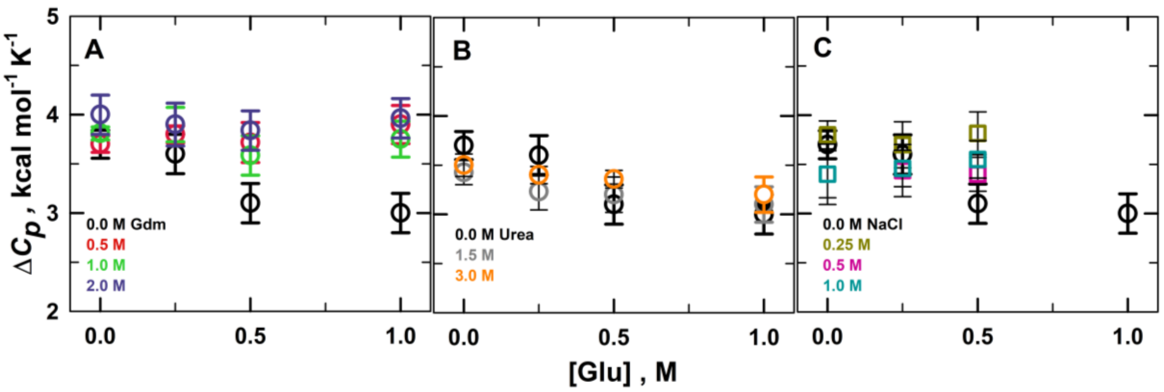

Figure 2.7. The heat capacity change of RNase A during the thermal denaturation in the presence and the absence of Gdm (A), urea (B), and NaCl (C) with increasing concentrations of Glu.

Similar to the effects observed in the case of RNaseA, Gdm could destabilize the intermediate and shift the unfolding to two-state transition in α-LA as well (Figure 2.8A-D). However, in contrast to RNase A, even lower concentrations of urea could destabilize the thermal-equilibrium intermediate of α-LA (Figure 2.8E-H). The extent of decrease in the stability of the protein was less in urea compared to the ionic denaturant, Gdm. The addition of salt also disrupted the formation of intermediate; however, it could slightly stabilize the protein against thermal denaturation (Figure 2.8E-G). The denaturation transitions were fitted to equation 1 (two-state) and the parameters are presented in Figures 2.9 & 2.10 and in Table 2.3.

Table 2.3. Thermal denaturation of α-LA in denaturants and salt[§]

Cosolvent	**No Glu**		**0.25 M Glu**		**0.5 M Glu**		**1.0 M Glu**	
	T_m	ΔH	T_m	ΔH	T_m	ΔH	T_m	ΔH
Gdm								
0.25 M	328.4± 0.0	55.5±0.9	336.0±0.1	75.0±1.4	339.2±0.1	82.0±1.8	342.0±0.2	86.5±2.1
0.5M	325.0±0.2	53.5±2.6	334.3±0.2	75.0±1.8	337.6±0.2	78.0±1.3	341.7±0.3	79.9±1.8
1.0M	317.1±0.5	40.9±2.9	330.3±0.1	65.6±1.5	333.9±0.2	72.3±2.0	337.8±0.3	74.7±1.8
Urea								
0.5M	325.8±0.1	50.2±1.0	336.1±0.1	67.7±1.2	338.8±0.2	87.0±2.3	342.2±0.3	96.9±2.8
1.0M	323.7±0.2	45.2±1.2	334.5±0.4	62.0±2.8	337.0±0.1	72.4±1.4	341.2±0.3	86.5±2.6
1.5 M	321.2±0.2	41.8±1.4	332.3±0.2	58.0±2.5	334.9±0.1	67.6±1.0	337.4±0.3	72.5±2.3
Salt								
0.25 M	329.5±0.4	63.6±3.7	337.0±0.2	79.0±3.4	340.3±0.2	100.0±3.8	-	-
0.5 M	329.5±0.3	61.1±3.1	339.5±0.2	80.0±3.6	342.5±0.4	108.4±2.9	-	-
1.0 M	332.0±0.2	61.9±1.8	340.0±0.3	82.0±2.1	344.6±0.4	108.0±2.8	-	-

[§] T_m in kelvin and ΔH in kcal/mol units

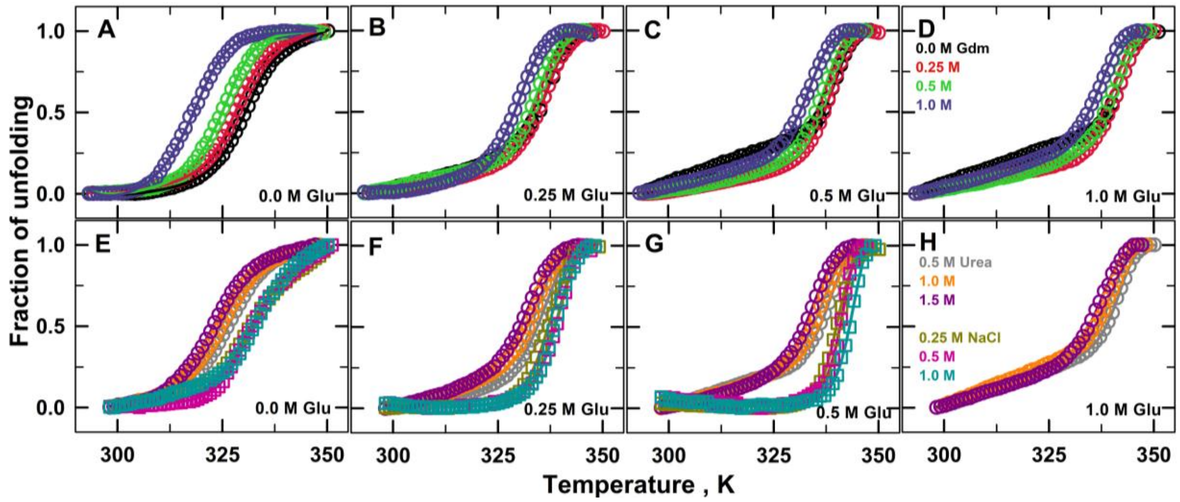

Figure 2.8. The effect of Gdm (upper panels), urea and salt (lower panels) on the thermal denaturation of α-LA in varying concentrations of Glu monitored using absorbance changes. The color codes represent the concentrations of cosolvents as mentioned in the figure labels. The solid lines represent the data fitted using equation 1 or 2. The thermodynamic parameters obtained are presented in Table 2.3.

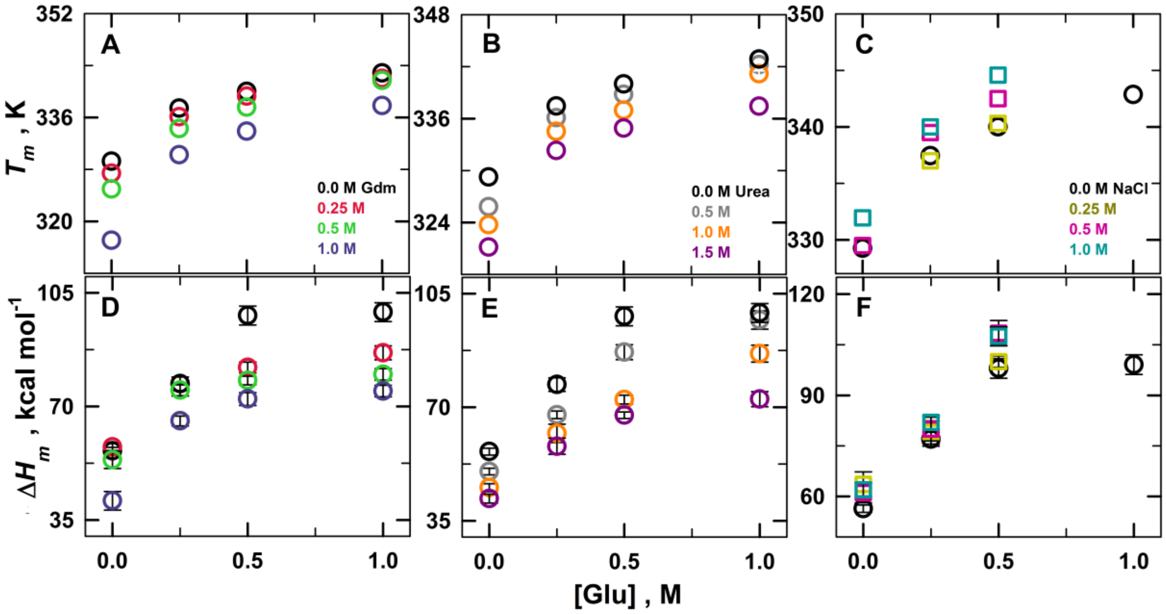

Figure 2.9. Changes in transition midpoint of thermal denaturation, T_m (upper panels) and enthalpy of unfolding, ΔH_m (lower panels) of α-LA in Glu upon the addition of varying concentrations of Gdm (A and D), urea (B and E), and salt (C and F). The color codes represent the concentrations of cosolvents as mentioned in the figure labels. In the case of three-state transitions, the parameters of the major transition (I ↔ U) are plotted.

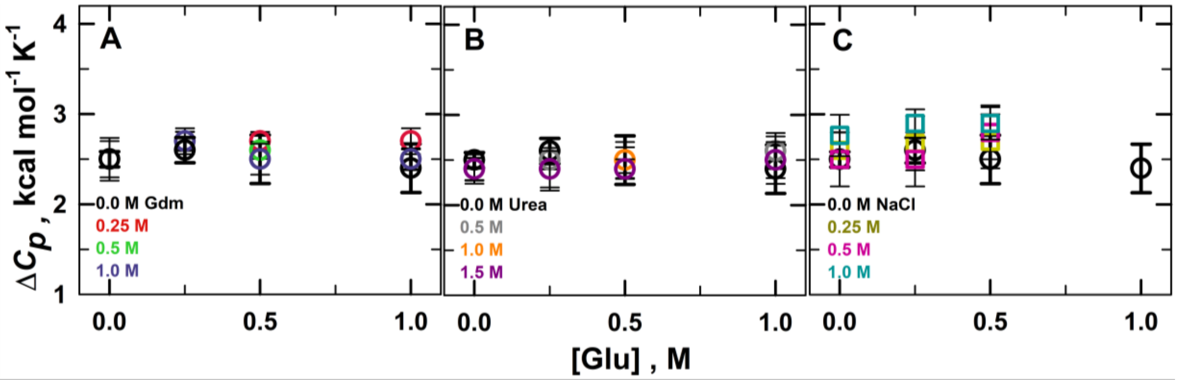

Figure 2.10. The heat capacity change of α-LA during thermal denaturation in the presence and the absence of Gdm (A), urea (B), and NaCl (C) with increasing concentrations of Glu.

2.4.3. *Chemical denaturation studies*:

The thermodynamic stability provided by Glu was further assessed by chemical denaturation of the proteins using Gdm at five different temperatures, 293, 298, 303, 308 & 313 K. In this temperature range, the fractions of unfolding of both the proteins were minimal in the absence of any cosolvent (Figure 2.1). RNase A followed two-state unfolding transitions at all the five temperature conditions when denatured with Gdm. Addition of Glu did not alter the two-state transition (Figure 2.11). All the transition curves were fitted using equation 3 and the calculated free energies of unfolding (ΔG_{unf}) and C_m values are presented in Figure 2.12 and Table 2.4. The slope of the transition region (m_g values) which represents the dependence of ΔG on the concentration of denaturant slightly increased at higher concentrations of Glu but reduced with an increase in temperature (Figure 2.13).

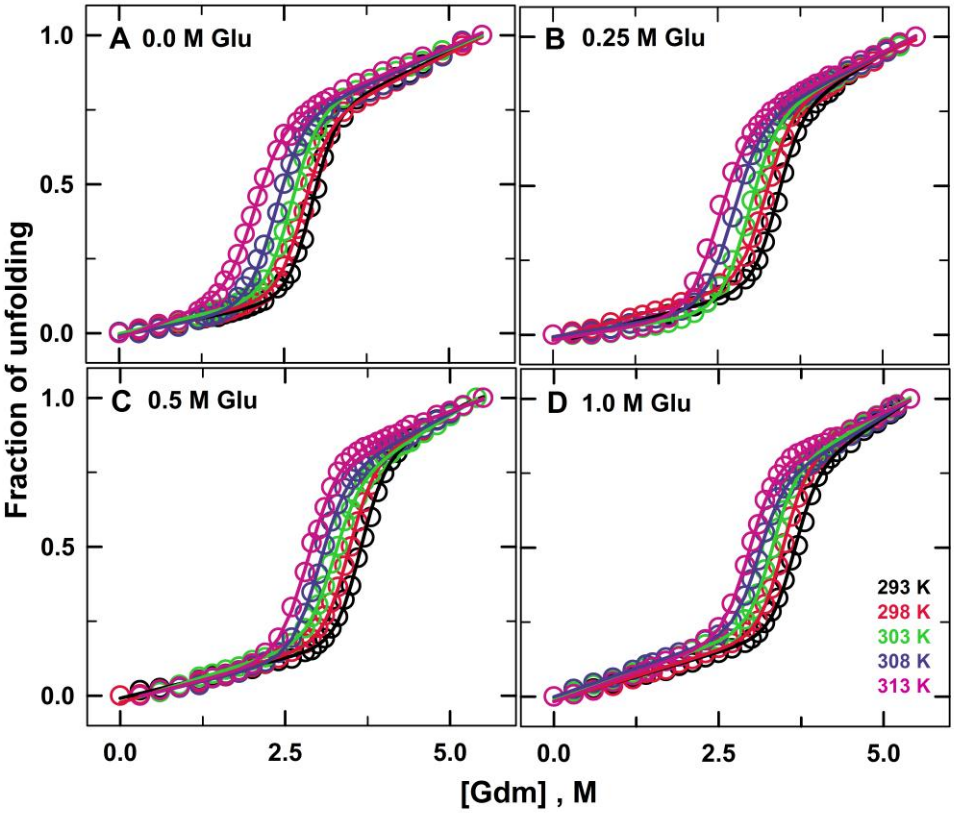

Figure 2.11. Gdm induced unfolding of RNase A in the absence (A) and the presence of 0.25 M (B), 0.5 M (C) and 1.0 M (D) of Glu. The color codes represent different experimental temperatures ranging from 293 to 313 K as mentioned in the figure label. The solid lines represent the data fitted for two-state unfolding transition using equation 3. The parameters obtained are presented in Table 2.4.

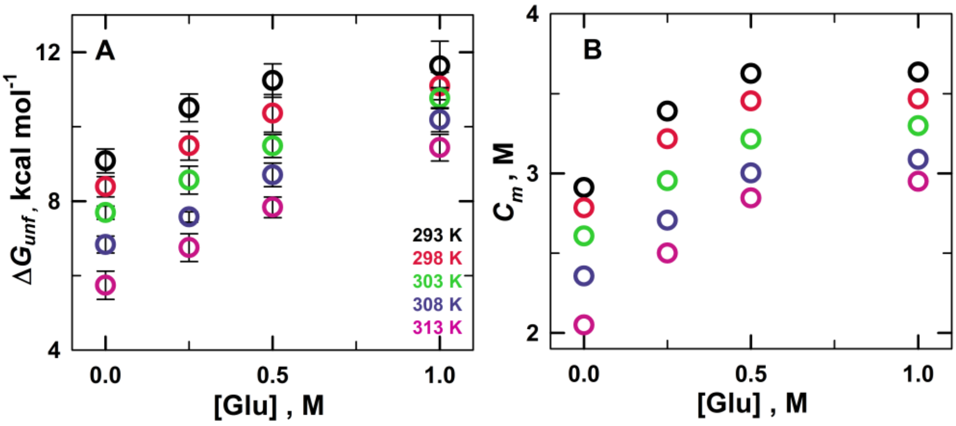

Figure 2.12. Change in free energy of unfolding (A) and C_m-values (B) of the Gdm-induced unfolding of RNase A in varying concentrations of Glu. The color codes represent different experimental temperatures ranging from 293 to 313 K as mentioned in the figure label.

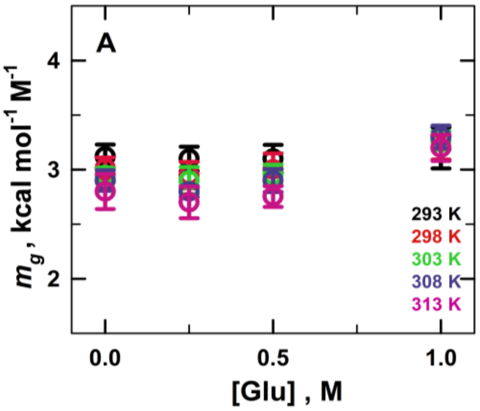

Figure 2.13. The slope of Gdm-induced chemical denaturation transitions (m_g-values) of RNase A in the presence of different concentrations of Glu measured between 293 K and 313 K with the interval of 5 K.

Table 2.4. Gdm-induced denaturation of RNase A at different temperatures

Temperature/ [Glu]	**293K**	**298K**	**303K**	**308K**	**313K**
			ΔG_{unf} (kcal mol^{-1})		
0 M	9.1±0.3	8.4±0.3	7.7±0.2	6.8±0.2	5.7±0.4
0.25 M	10.5±0.4	9.5±0.4	8.6±0.4	7.6±0.1	6.8±0.4
0.5 M	11.2±0.5	10.4±05	9.5±0.3	8.7±0.3	7.8±0.3
1.0 M	11.6±0.7	11.1±0.4	10.8±0.3	10.2±0.3	9.4±0.4
			m_g (kcal mol^{-1} M^{-1})		
0 M	3.1±0.1	3.0±0.1	3.0±0.1	2.9±0.1	2.8±0.1
0.25 M	3.1±0.1	2.9±0.1	2.9±0.1	2.8±0.1	2.7±0.1
0.5 M	3.1±0.1	3.0±0.1	2.9±0.1	2.9±0.1	2.8±0.1
1.0 M	3.2±0.2	3.2±0.1	3.3±0.1	3.3±0.1	3.2±0.1
			C_m (M)§		
0 M	2.9	2.8	2.6	2.4	2.1
0.25 M	3.4	3.3	3.0	2.7	2.5
0.5 M	3.6	3.5	3.2	3.0	2.8
1.0 M	3.6	3.5	3.3	3.1	2.9

§ Standard error in $C_m \leq 0.05$

In the case of α-LA as well, the addition of Glu did not affect the two-state unfolding transitions at all the studied temperatures (Figure 2.14). Also, the unfolding free energy of α-LA increased with the increasing concentrations of Glu and decreased with increasing temperature. The same trend was observed with C_m values as well (Figure 2.15 and Table 2.5). The m_g values slightly increased as the concentration of Glu was increased, but decreased when the temperature was increased (Figure 2.16A).

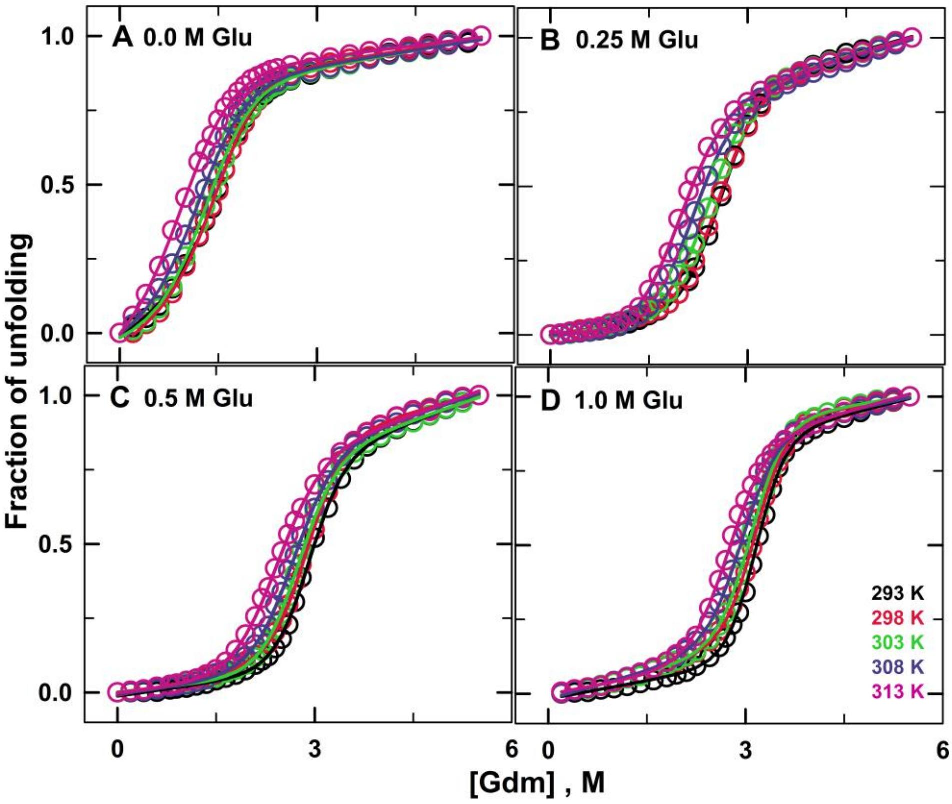

Figure 2.14. Gdm induced unfolding of α-LA in the absence (A) and the presence of 0.25 M (B), 0.5 M (C) and 1.0 M (D) of Glu measured at different temperatures ranging from 293 to 313 K. The solid lines represent data-fit for two-state unfolding transition using equation 3. The parameters obtained are presented in Table 2.5.

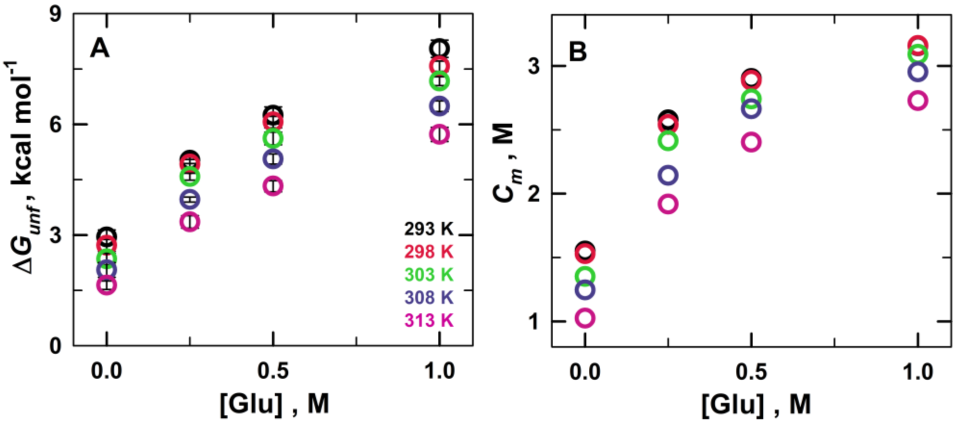

Figure 2.15. Change in free energy of unfolding (A) and C_m-values (B) of the Gdm-induced unfolding of α-LA in varying concentrations of Glu measured at different temperatures ranging from 293 to 313 K.

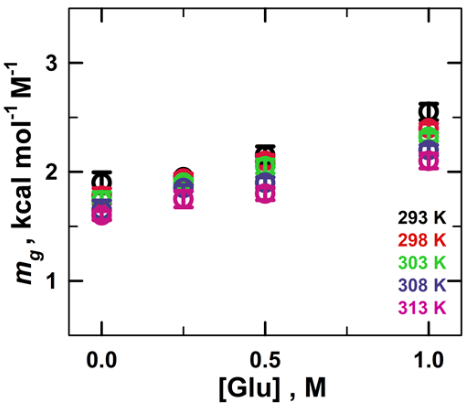

Figure 2.16. The slope of Gdm-induced chemical denaturation transitions (m_g-values) of α-LA in the presence of different concentrations of Glu measured between 293 K and 313 K with the interval of 5 K.

Table 2.5. Gdm-induced denaturation of α-LA at different temperatures

Temperature/ [Glu]	**293K**	**298K**	**303K**	**308K**	**313K**
			ΔG_{unf} (kcal mol^{-1})		
0 M	2.9±0.2	2.7±0.2	2.3±0.2	2.1±0.2	1.6±0.1
0.25 M	5.0±0.1	4.9±0.1	4.6±0.1	4.0±0.1	3.4±0.2
0.5 M	6.2±0.2	6.1±0.2	5.6±0.2	5.1±0.1	4.3±0.1
1.0 M	8.0±0.2	7.6±0.1	7.2±0.1	6.5±0.1	5.7±0.2
			m_g (kcal mol^{-1} M^{-1})		
0 M	1.9±0.1	1.8±0.1	1.7±0.1	1.7±0.1	1.6±0.0
0.25 M	2.0±0.0	1.9±0.0	1.9±0.0	1.9±0.0	1.8±0.1
0.5 M	2.2±0.1	2.1±0.1	2.1±0.1	1.9±0.1	1.8±0.1
1.0 M	2.6±0.1	2.4±0.0	2.3±0.0	2.2±0.0	2.1±0.0
			C_m (M)[§]		
0 M	1.6	1.5	1.4	1.2	1.0
0.25 M	2.6	2.5	2.4	2.2	1.9
0.5 M	2.9	2.9	2.7	2.7	2.4
1.0 M	3.2	3.2	3.1	3.0	2.7

[§] Standard error in $C_m \leq 0.05$

2.4.4. Fluorescence quenching and near UV-CD:

The extent of solvent exposure of the buried residues in the presence of Glu was probed by the ability of acrylamide to quench the fluorescence emission of aromatic residues. Addition of Glu decreased the effective quenching of acrylamide which resulted in a decrease in $K_{S\text{-}V}$ values for both RNase A and α-LA (Figure 2.17A). The reduction in $K_{S\text{-}V}$ was found to be more in the case of RNase A compared to α-LA. Moreover, the tertiary structural changes monitored using near UV-CD spectra (Figure 2.17B and C) suggested that the presence of Glu did not significantly alter the tertiary interactions in both the proteins.

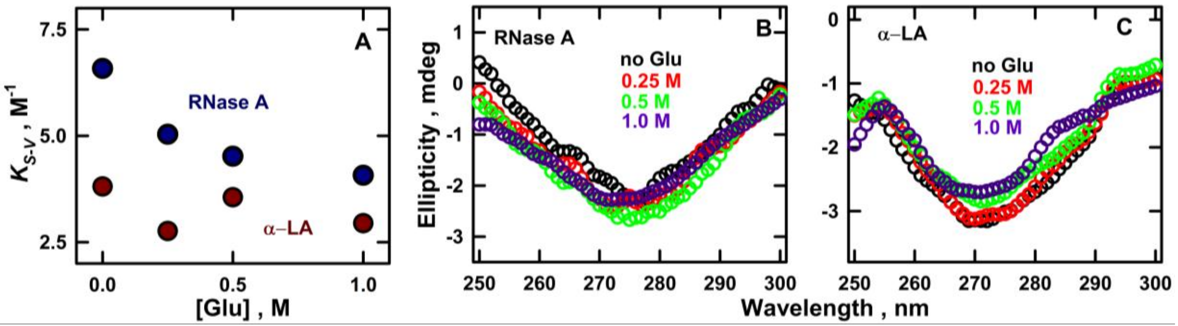

Figure 2.17. (A) Fluorescence quenching effect of acrylamide on RNase A (blue) and α-LA (red) in the presence of different concentrations of Glu measured as Stern-Volmer constant ($K_{S\text{-}V}$). Near-UV circular dichroism spectra of RNase A (B) and α-LA (C) measured in the absence (black) and the presence of 0.25 (red), 0.5 (green), and 1.0 M (purple) Glu.

2.4.5. Volumetric analysis:

As Glu was found to exert stabilization on both the proteins, it would be interesting to analyze the effect of this amino acid on partial molar volume and compressibility of the proteins. This could provide the essential information on changes in the intrinsic volume and the hydration of the proteins upon addition of Glu. The partial molar volumes (V^o) of both RNase A and α-LA were decreased as the concentration of Glu was increased in the solution (Figure 2.18A-B). The increase in temperature of measurement from 293 K to 313 K showed an increase in V^o values at all the concentrations of Glu. The V^o values were also measured at a slightly higher temperature (328 K) where the intermediate population could be more. Due to experimental limitations, the measurements could not be performed above 328 K. The adiabatic compressibilities (K_s) of the proteins were also decreased with increasing concentrations of Glu (Figure 2.18C-D). However, increasing the solution temperature decreased the K_s values of both the proteins and the extent of decrease was reduced at higher Glu concentrations.

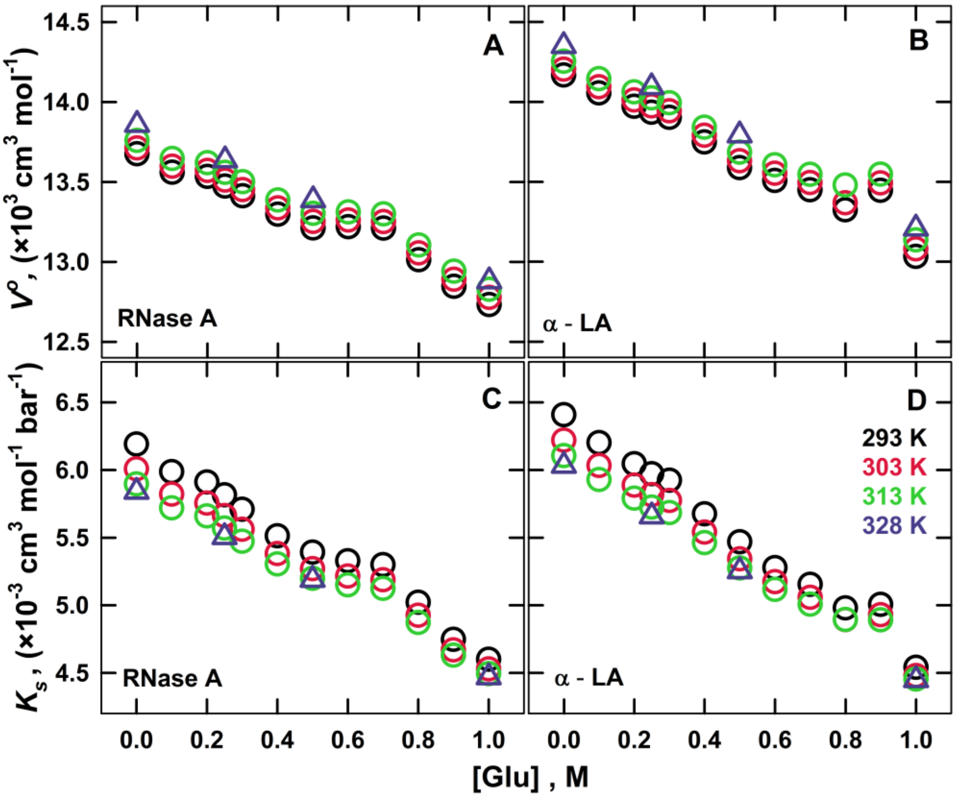

Figure 2.18. Partial molar volumes (A and B) and adiabatic compressibilities (C and D) of RNase A and α-LA evaluated from density and acoustic measurements of the proteins in different concentrations of Glu at 293 (black circles), 303 (red circles), 313 (green circles), and 328 K (purple triangles).

2.5. Discussion:

The stability of proteins rely upon the balance between electrostatic, hydrophobic, van der Waals, and hydrogen bonding interactions which determine their packing and hydration properties in solvents.[58-60] Living organisms use small osmolyte molecules to alter this balance in order to counteract their environmental stress. Glutamate, a negatively charged amino acid, is one such naturally occurring osmolyte which has been less understood on its ability to modify the stability of proteins.

2.5.1. Glu induces a thermal denaturation intermediate:

RNase A and α-LA show two-state transition without any detectable intermediate during their thermal denaturation.[61-62] Addition of Glu causes two major changes in their thermal denaturation process: (a) it stabilizes the proteins against temperature, and (b) it induces an early equilibrium intermediate at higher concentrations (≥ 0.5 M). The transition midpoint (T_{m1}) and change in enthalpy (ΔH_{m1}) for the first transition are similar at different temperature and the enthalpy of unfolding also increases for the second transition (T_{m2} and

ΔH_{m2}, respectively). This 〝major-transition〞 is close to the unfolding curves obtained with two-state transitions, thus could be assumed to represent intermediate ↔ unfolded. Though some of the other osmolytes including amino acids and their derivatives such as glycine, proline, and sarcosine are also known to stabilize these proteins,[35,61-65] none of these osmolytes induce three-state thermal unfolding in either of the proteins. Further, the extent of stabilization provided by Glu is higher than the other amino acid osmolytes so far reported. Lysine is shown to provide more thermal stability with the increase in T_m by ~ 5.5 K in RNase A and by ~9.7 K in α-LA.[65] For more insight on the extent of stability provided by the Glu against thermal unfolding, $\Delta\Delta G_T$ values (the free energy of unfolding calculated at the midpoint temperature, T_m of 338 K for RNase A and 329 K for α-LA, where unfolding free energy is zero in the absence of Glu) are analyzed. From Figure 2.19, it is evident that Glu exerts nearly 3.0 – 3.2 kcal/mol of additional stability to the proteins. It may also be noted that α-LA acquires slightly higher stability than RNase A at all the studied Glu concentrations.

Further, the V^o values are reduced upon increasing Glu concentration for both the proteins (Figure 2.18). The changes in conformational states of proteins involve changes in intrinsic volume and surface hydration of the proteins which in turn would affect the partial molar volume and compressibility of the proteins. The decrease in V^o could be due to either cosolvent induced reduction in the internal cavity, thus, the intrinsic volume of the protein, or due to an increase in hydration around the protein, or the combined effect of both.[66-67] The increase in hydration is generally expected by an increase in the solvent accessible surface area of the protein or preferential hydration of water molecules on the protein's surface in the presence of a cosolvent. Near-UV CD experiments and the $K_{S\text{-}V}$ values obtained from fluorescence quenching (Figure 2.17) suggest that the proteins become compact and the hydrophobic (aromatic) residues are not exposed to the solvent upon addition of Glu in both RNase A and α-LA. Therefore, the changes in V^o could not be attributed to the increase in solvent accessible surface area of the protein; thus, it might arise from the decreased intrinsic volume of the protein by increased compactness and preferential hydration of water molecules in the presence of Glu as found in the cases of other stabilizing osmolytes such as sucrose and sorbitol.[68-69] These changes are further complemented with the reduction in adiabatic compressibility (K_S) upon increasing Glu (Figure 2.18) which also supports the possibility of an increase in compactness of the proteins. As the temperature increases, the V^o values increase probably due to the increase in the intrinsic volume of the proteins at elevated temperature and such an increase is uniform throughout the Glu concentration. The K_s values nevertheless come down at higher temperatures, which might indicate the increase in hydration of the interior of the protein which becomes relatively accessible to the solvent molecules at elevated temperatures. This effect could overcome the positive contribution of an increase in cavities in the case of compressibility. Further, the addition of Glu reduces the extent of decrease in compressibility at higher temperature and almost nullifies at the concentration of 1 M. This suggests that the presence of Glu reduces the internal fluctuations in the proteins at higher temperature.

Though Glu is a charged amino acid, it shows a similar effect on the proteins with positive (RNase A) and negative (α-LA) surface charges (Figure 2.20) at neutral pH. This suggests that the contribution of direct electrostatic interaction between the protein surface and Glu is minimal and the amino acid acts through its ability to change the water structure around the protein, which is generally known as preferential hydration effect.

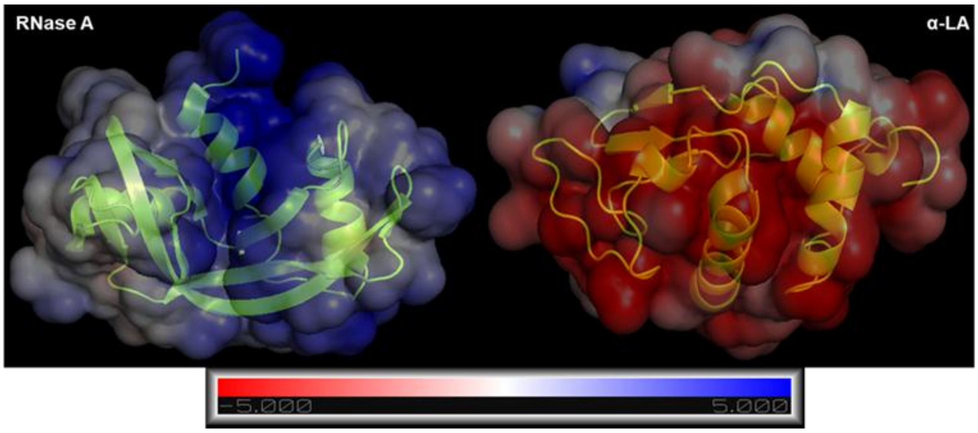

Figure 2.20. The charge distribution on the surface of RNase A (A) and α-LA (B) where red indicates negative charges, blue indicates positive charges and white indicates non-polar regions. The surface potential maps were generated in Pymol using Adaptive Poisson Boltzmann Solver (ABPS).

However, there could be a trivial effect of charge-charge interactions which might slightly alter the extent of stability offered by Glu. Similar preferential hydration effect has been found with other amino acids induced stabilization or destabilization of proteins[37,65,70-71] and with many other osmolytes as well.[72-73]

The transfer free energy studies suggest that the interaction between osmolytes and protein backbone contributes largely to the free energy difference between the native and unfolded states, though the backbone atoms occupy only one quarter of the total surface area of the proteins.[74] It is also proposed that osmolytes-induced changes in the hydration of protein’s surface are majorly determined by the solvation around the backbone.[75] In addition, increase in the fraction of polar surface area of osmolyte is expected to decrease the transfer free energy of proteins and to reduce the stabilization effect.[76] In the present case as well, Glu might increase the solvation of the protein backbone, which is similar for both the proteins. Also, the transfer free energy of the proteins would be positive in the presence of Glu according to Street *et al*,[76] since the osmolyte has fractional polar surface area <0.6. These observations emphasize that preferential solvation and transfer free energy of protein's backbone could be the major determinants of the osmolyte-induced stability.

2.5.2. Effect of denaturants and salt on the intermediate:

The factors stabilizing the intermediate structure could be understood by the effect of added cosolvents on the stability of the intermediate. Addition of Gdm destabilizes the formation of the intermediate and both the proteins adapt two-state thermal denaturation. However, urea could not completely transfer the thermal denaturation into a two-state process in the case of RNase A (Figure 2.5), particularly in the presence of higher concentration of Glu. The denaturation effect of urea involves breaking down of only hydrogen bonding interactions, whereas Gdm can disrupt the electrostatic interactions as well due to its ionic nature.[77] Thus, the results propose that electrostatic interactions might also contribute to the stabilization of the intermediate apart from the hydration changes discussed in the previous section. Urea could completely destabilize the intermediate formed by α-LA (Figure 2.8) which could be due to the weak electrostatic interactions between the protein and Glu, as α-LA is an acidic protein. The addition of salt which could prevent the formation of thermal equilibrium intermediate further supports the contribution of electrostatic interactions. The change in T_m values also suggests that the destabilization of the proteins by urea is less than Gdm and the extent of decrease in ΔH_m values is less in the case of urea. This again emphasizes on the contributions from ionic interactions in Gdm-induced denaturation.

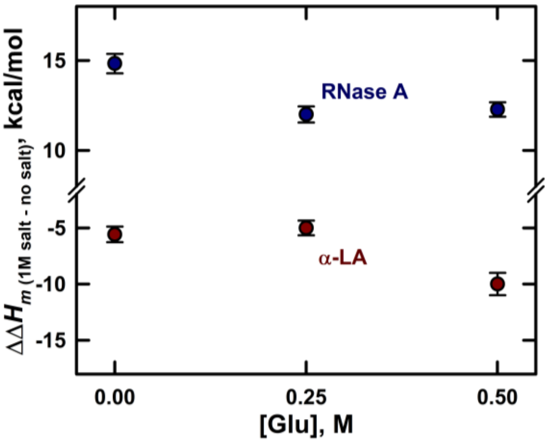

Figure 2.21. The difference between the ΔH_m values of the proteins in the presence and the absence of 1 M salt measured in different concentrations of Glu. The ΔH_m values were obtained from thermal denaturation studies, tabulated in Tables 2.2 and 2.3.

The effect of salt on the thermal stability of the proteins is different from that of the denaturants. Addition of salt slightly increases the T_m values of both the proteins, thus suggesting a stabilization effect against temperature. The difference in ΔH_m of unfolding calculated in the presence and the absence of the salt (Figure 2.21) indicates that the salt-induced stabilization is entropy-driven in RNase A but enthalpy-driven in α-LA. Addition of Glu further increases the stability through its enthalpic effect.

Further, the heat capacity is decreased for the “major-transitions” in RNase A where Glu induces a thermal-equilibrium intermediate (Figure 2.7A). Though the stabilizing osmolytes could increase ΔC_p,[78] this could not be expected in the present case due to the formation of an intermediate during the unfolding. The decrease in ΔC_p suggests that the exposure of non-polar residues during N → U is marginally higher than I → U transition of RNase A upon heating. The addition of Gdm and salt does not show any significant change in ΔC_p in the absence or in the presence of Glu (Figure 2.7), which might be due to their destabilizing nature of the intermediate. However, in the presence of urea, ΔC_p is comparable with the values observed for major-transitions in three-state unfolding conditions, since urea poorly destabilizes the intermediate formed by RNase A. In the case of α-LA, there is no detectable change in ΔC_p values (Figure 2.10) between the two-state denaturation ($\leq$ 0.25 M Glu) and the “major-transition” in three-state denaturation ($\geq$ 0.5 M Glu). This suggests that the amount of exposure of hydrophobic residues during unfolding is similar for both the native and intermediate states in α-LA.

2.5.3. Effect of Gdm on the Glu-induced stability:

Since Glu stabilizes the proteins against thermal denaturation, its role during chemical denaturation of the proteins at different temperatures is also analyzed. Glu stabilizes both the proteins against Gdm-induced denaturation, and all of the unfolding follow two-state transitions. However, the stabilization effect varies with temperature. Further, the additional stability acquired by the protein in the presence of Glu was calculated as the difference in unfolding free energy in the presence and the absence of Glu, $\Delta\Delta G_{unf}$ (Figure2.22). In the case of RNase A, Glu stabilizes the protein more effectively at higher temperature whereas in α-LA, the extent of stability is reduced at higher temperature. The extent of stability calculated as ΔG_{unf} per mole of Glu confirms the same (Figure 2.22C). This is in contrast to the effect shown by Glu in thermal denaturation wherein α-LA is stabilized more. This suggests that the mechanism of Glu-induced stabilization of the proteins against thermal and chemical perturbations might be different.

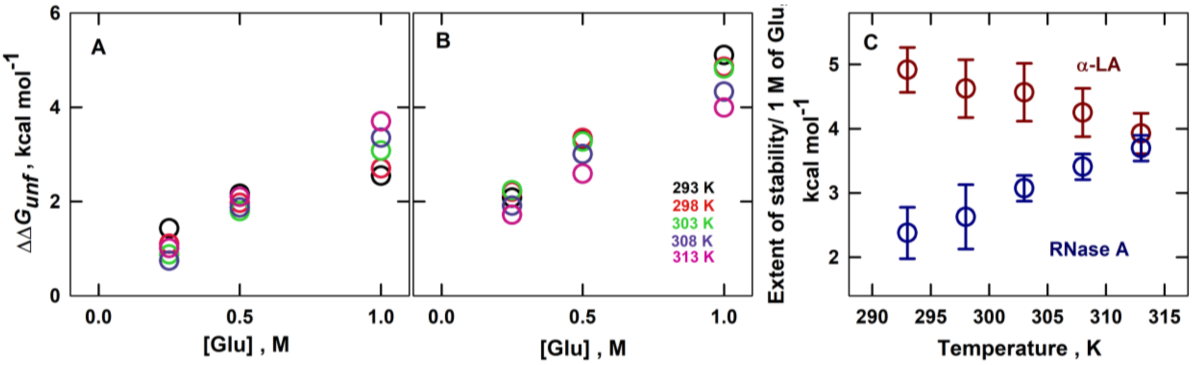

Figure 2.22. (A) and (B) The extent of stabilization of RNase A (A) and α-LA (B) with the increasing concentration of Glu calculated as the difference between the free energies of unfolding in the presence and the absence of Glu measured at varying temperatures between 293 K and 313 K. (C) Extent of stability obtained per mole of Glu by RNase A (blue) and α-LA (red) at different temperatures derived from isothermal Gdm-induced unfolding transitions (refer Figures 2.11 & 2.14).

The addition of Glu increases the m_g values of denaturation transitions. The m_g values represent the extent of unfolding of the protein in its denatured state.[79] The increase in m_g values suggests that the denatured states of RNase A and α-LA are more extended in the presence of Glu. In case of glutaminyl-t-RNA synthetase also the urea-induced denaturation shows an increase in m_g value, thus an increase in the solvent exposure of its unfolded state, in the presence of Glu compared to the state in its absence.[38] Further, increasing temperature marginally decreases the m_g values. It is generally expected that raising the temperature would increase the hydrophobic exposure of the protein interior, thus, might increase m_g value. Also, some proteins show a temperature independence of m_g values at least up to 323 K.[47] Moreover, the general trend of positive correlation between m_g values and the ΔC_p values could not be observed in the present case, because thermal denaturation follows three-state transitions whereas Gdm-induced unfolding follows two-state transitions for both the proteins.

2.6. *Summary*:

The effect of a charged amino acid osmolyte, Glu on RNase A and α-LA is examined. Glu stabilizes the proteins against temperature and the extent of stability is slightly higher for α-LA. Glu also induces an equilibrium intermediate during thermal denaturation at the concentrations ≥0.5 M. Addition of Gdm and urea could destabilize the intermediate and it is found to be enthalpy-driven. The intermediate is majorly stabilized by preferential hydration of water around the proteins, though ionic interactions might also contribute to a certain extent. Glu decreases the intrinsic volume of the proteins and also reduces the internal fluctuations at higher temperature. Salt-induced stabilization of the proteins is slightly different between RNase A (entropy-driven) and α-LA (enthalpy-driven). The counteracting effect of Glu against chemical denaturation of the proteins by Gdm is also investigated. Glu stabilizes RNase A more effectively at higher temperature. However, the stabilization exerted by the osmolyte on α-LA is reduced at higher temperature. The study also indicates that Glu-induced stabilization of the proteins against thermal and chemical denaturation processes might follow different mechanisms.

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Chapter 3

Effect of Charged-Amino acids on the Stability of Proteins

1. Abstract:

Studies on the osmolyte-induced effects on proteins help in enhancing protein stability under stressed conditions for various applications. The present study examines the effect of charged amino acids Arg, Asp, and Lys on the stability of RNase A and α-LA. The thermal stabilities of the proteins in the presence of osmolytes are monitored by spectral changes and the structural changes are analyzed using fluorescence quenching and near-UV circular dichroism (CD). These results are compared with the previous chapter on the effect of Glu. Arg destabilizes the proteins whereas Asp and Lys and Glu stabilize the proteins. The extent of stability provided by Asp and Glu during thermal denaturation is almost same and higher than Lys in RNase A. However, the stability acquired in the presence of Asp and Lys is comparable for α-LA and Glu provides higher stability. Similar to Glu, Asp and Lys also exhibit enthalpy-driven protein stabilization. The volumetric and compressibility measurements of both the proteins in the presence of Arg, Asp and Lys suggest that the proteins attain a more compact state. Further, the fluorescence quenching and CD results show that the addition of amino acids does not alter the structure of the proteins significantly. In addition, guanidinium chloride-induced denaturation of both the proteins at different temperatures in the absence and the presence of Arg, Asp and Lys were carried out. These results complement with the observations of thermal denaturation studies. Arg is found to be effective at destabilizing RNase A compared to α-LA. Similarly, Lys and Asp stabilizing efficiency is more for RNase A than α-LA. In RNase A, the extent of stabilization by all the amino acids is higher at higher temperatures and in α-LA, although trivial, all the amino acids show slightly higher stabilization at lower temperatures. Thus, these results suggest that Arg, Asp, and Lys act in a concentration- and temperature-dependent manner irrespective of the surface charge on the proteins.

3.2. Introduction:

Amino acids are widely used in biotechnology industries to enhance the solubility and foldability of proteins along with other osmolytes such as sugars and polyols.[1-2] They are also used as pharmaceutical excipients in therapeutic protein formulations[3-4] and to enhance the protein recovery from various chromatographic columns.[5-6] Amongst the naturally occurring amino acids, the charged amino acids have certain advantages due to their charge-charge (preferential) interactions with proteins. At lower concentrations, charged residues do not affect the thermodynamic stability of proteins. At the concentrations greater than 0.1 M, they significantly change the stability either through strong preferential interaction or solvation.[7-8]

Arginine (Arg) is a positively charged amino acid residue with a propyl guanidino side chain. It is extensively used as a cosolvent with a broad spectrum of applications from laboratories to industries.[9-11] For instance, arginine is used to enhance the refolding proficiency of pharmaceutically important proteins from the inclusion bodies,[12-13] supplemented as an excipient in the production of monoclonal antibodies,[10] and added as cryo- and lyo-preservative during freeze-drying.[14-16] However, Arg has certain limitations due to its destabilizing effects on folding equilibrium which could be attributed to the guanidino side chain similar to that of guanidine hydrochloride (Gdm).[11, 17-18] Studies have shown that Arg reduces the thermal stability of proteins.[17,19] However, increasing surface tension and steric repulsion effects of its side chain makes it a weaker denaturant.[1,16] Arg reduces the stability and activity of enzymes such as aminoacylase, pyruvate kinase, lactate dehydrogenase and α-crystallin.[19-21] Arg is also found to suppress the aggregation of proteins such as hen egg white proteins and phosphoryl kinase by interacting with their aromatic residues which are known to drive protein-protein interactions.[11,22-23] Molecular simulation studies on insulin and lysozyme also support that arginine interacts specifically with aromatic and acidic groups of the proteins.[24] In the process of suppression of aggregation, Arg is found to solubilize the oligomers in the aggregation pathway of IgG.[19,25] Therefore, Arg is defined to exert "dual nature" of both destabilizer of native proteins and suppressor of aggregation.

In contrast to Arg, lysine (Lys) might either stabilize or have a little/no effect on protein stability as an additive. Lys is found to increase the thermal stability of lysozyme, myoglobin, cytochrome *c* and tubulin,[17,26-27] but destabilizes RNase A at lower concentrations at pH 5.[26,28] Lysine is also used as an additive to protect proteins such as ciliary neurotrophic factor (CNTF) against heat stress during storage and transport and with combination of other osmolytes to protect from mechanical stress as well.[29] Apart from the positively charged Arg and Lys, negatively charged amino acids aspartate (Asp) and glutamate (Glu) are also known for their osmolytic activities.[30] *In vitro* experiments show that Glu stabilizes globular proteins and could induce equilibrium intermediate against thermally induced stress and counteract the chemical denaturation of proteins against GdmCl and urea.[8] However, the effect of Glu on the self-aggregation of proteins is stress-specific. Glu suppresses the aggregation of lysozyme against thermal stress whereas enhances the aggregation during the dilution from urea-denatured state.[11] The effect of Asp on proteins is found to be concentration-dependent. Asp induces reversible unfolding of creatine kinase and partially dissociates its dimers.[31] In the case of aminoacylase, Asp denatures the protein at lower concentrations whereas induces molten-globule like intermediate at higher concentrations.[32]

Here, we examine the effects of charged amino acids Arg, Asp, and Lys on two globular proteins, ribonuclease A (RNase A) and alpha-lactalbumin (α-LA). These two model proteins differ in their surface charge distribution (predominantly positively and negatively charged, respectively). Thus, studies on these model proteins could provide a better understanding on whether the preferential interaction varies with the amino acids and the proteins' surface charge. Moreover, the stability of these proteins against temperature and chemical denaturant could be accurately measured by following the changes in absorbance and fluorescence emission of the proteins, respectively, upon denaturation without much interference from the osmolyte molecules.[26,33-34] This would aid to analyze the changes quantitatively in terms of thermodynamic parameters. The experimental results suggest that Arg destabilizes the proteins whereas Lys, and Asp stabilize the proteins. The structural and volumetric changes of the proteins in the presence of these amino acids are similar to that of Glu, (discussed in the previous chapter) emphasizing that the mechanism through which these amino acids act on the proteins could be common.

3.3. Materials and methods:

3.3.1. Materials:

L-arginine (Arg), L-lysine (Lys) and L-aspartate (Asp), sodium phosphate dibasic, sodium phosphate monobasic and guanidinium Chloride (Gdm) were purchased from SRL, India. Bovine pancreatic ribonuclease A (RNase A) and bovine α-lactalbumin (α-LA) were from Sigma.

3.3.2. Methods:

Thermal denaturation, chemical denaturation, structural and volumetric measurements were performed as described in the previous chapter in section 2.3.

3.4. Results:

3.4.1. Thermal denaturation in the presence of amino acids:

The model proteins, RNase A and α-LA, showed two-state transitions during their thermal unfolding with the T_m values of 338.2 (±0.1) and 329.2 (±0.1) K, respectively. The addition of Arg shifted the unfolding transitions of both the proteins towards lower temperature suggesting destabilization of the proteins by Arg (Figure 3.1A and 3.1D). The transitions curves were fitted using equation 2.1 and the parameters obtained are presented in Table 3.1. Increasing the concentration of Arg increased the extent of destabilization which was evident from the changes observed in T_m and enthalpy of unfolding (ΔH_m) of both the proteins (Figure 3.1). However, in the presence of Asp and Lys, the thermal transitions of both the proteins were shifted towards higher temperature suggesting stabilization of the proteins against temperature by Asp and Lys (Figure 3.2). In the case of RNase A, the stabilization effect of Asp was higher than that of Lys. In α-LA, both the amino acids showed an almost similar effect on T_m, however, the increase in ΔH_m value in the presence of Asp was more compared to Lys (Figure 3.3). The change in heat capacity (ΔC_p) was slightly decreased in Arg whereas Asp did not show significant changes in ΔC_p during thermal denaturation (Figure 3.4). In the presence of Lys, the ΔC_p of RNase A was marginally increased, but not in α-LA. All the calculated thermodynamic parameters are listed in Table 3.1.

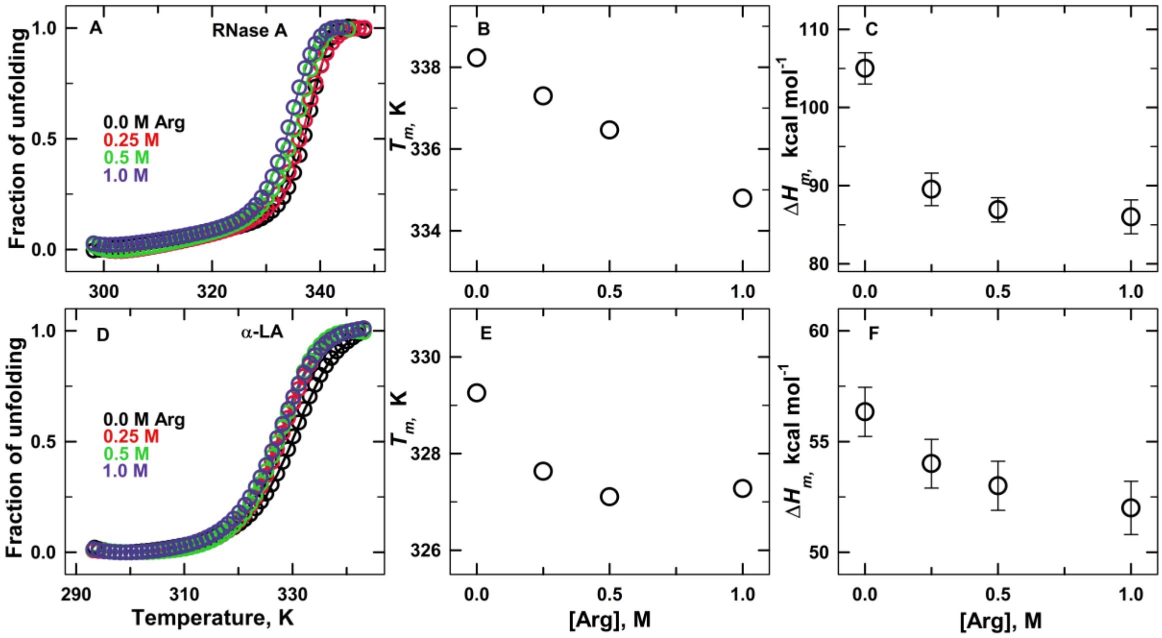

Figure 3.1. Thermal denaturation of (A) RNase A and (D) α-LA in the absence (black) and the presence of 0.25 (red), 0.5 (green), and 1.0 M (purple) of Arg. The solid lines represent the data fit with two-state assumption using equation 2.1. (B) & (C) are thermal denaturation midpoint (T_m) and enthalpy of unfolding (ΔH_m), respectively for RNase A and (E) & (F) are T_m and ΔH_m values, respectively for α-LA as calculated from their respective thermal transitions.

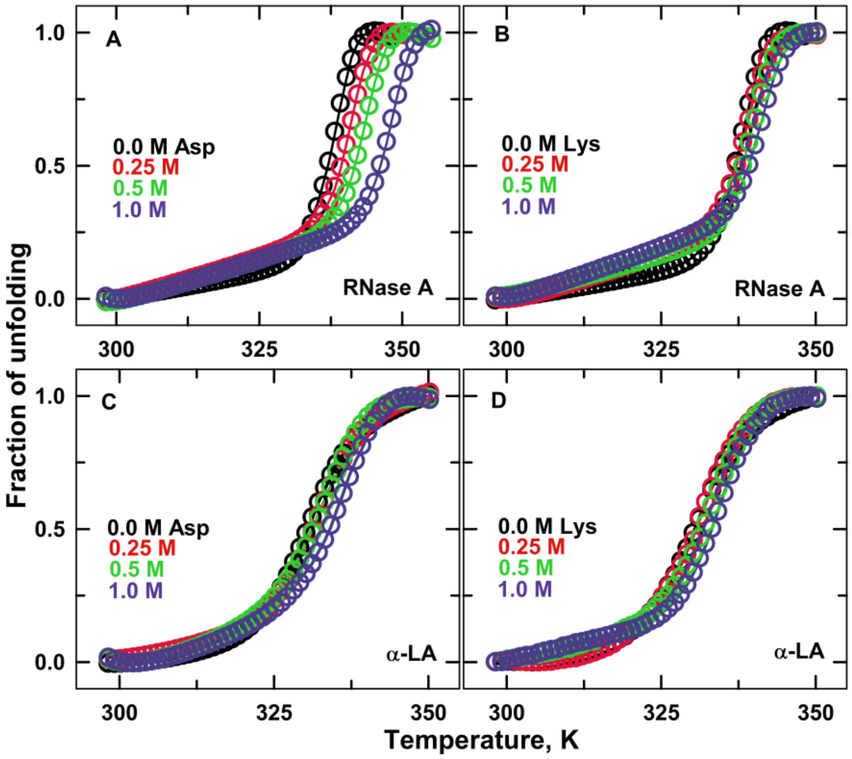

Figure 3.2. Thermal denaturation of RNase A in the presence of increasing concentrations of (A) Asp, and (B) Lys. (C) and (D) presents the thermal transition of α-LA in the presence of increasing concentrations of Asp and Lys, respectively. The solid lines represent the data fit using equation 2.1.

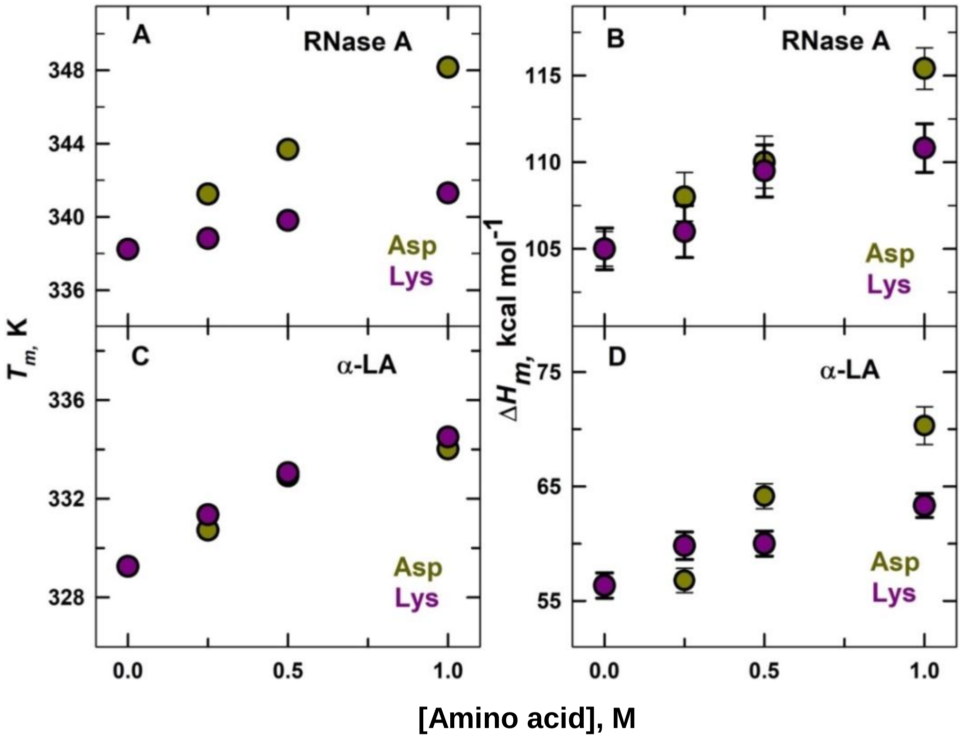

Figure 3.3. Thermal denaturation midpoint (T_m) and enthalpy of unfolding (ΔH_m) of (A & B) RNase A and (C & D) α-LA in the presence of increasing concentrations of Asp (green) or Lys (pink).

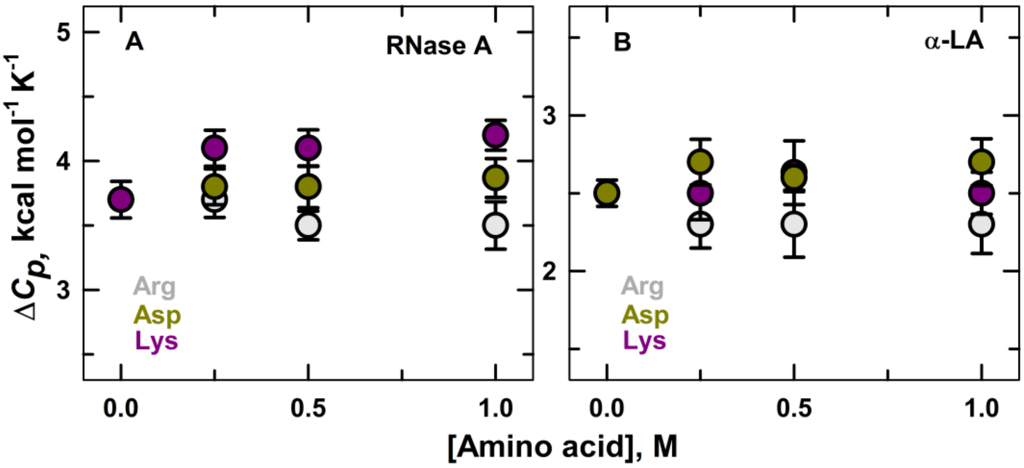

Figure 3.4. The heat capacity changes calculated from the thermal denaturation of (A) RNase A and (B) α-LA in the presence of varying concentrations of Arg (gray), Asp (green) and Lys (pink).

Table.3.1. Thermal denaturation of RNase A and α-LA in the presence of amino acids[§]

	[Cosolvent], M	T_m	ΔH_m	ΔC_p	T_m	ΔH_m	ΔC_p
			RNase A			**α-LA**	
	0.0	338.2±0.1	105.0±2.4	3.7±0.1	329.2±0.1	56.3±1.1	2.5±0.1
Arg	0.25	337.3±0.1	89.5±2.0	3.7±0.1	327.6±0.1	54.0±1.1	2.3±0.1
	0.5	336.4±0.1	86.9±1.5	3.5±0.1	327.1±0.1	53.0±1.1	2.3±0.2
	1.0	334.8±0.1	86.0±2.1	3.5±0.2	327.3±0.1	52.0±1.2	2.3±0.1
Asp	0.25	341.2±0.1	108.0±1.4	3.8±0.1	330.7±0.1	56.7±1.0	2.7±0.1
	0.5	343.7±0.1	110.0±1.5	3.8±0.1	332.9±0.1	64.1±1.0	2.6±0.1
	1.0	348.1±0.1	115.4±1.2	3.8±0.1	334.0±0.1	70.3±1.6	2.7±0.1
Lys	0.25	338.8±0.1	106.0±1.5	4.1±0.1	331.3±0.1	59.8±1.2	2.5±0.1
	0.5	339.8±0.1	109.5±1.5	4.1±0.1	333.0±0.2	60.0±1.1	2.6±0.2
	1.0	341.3±0.1	110.8±1.4	4.2±0.1	334.5±0.2	63.3±1.0	2.5±0.1

[§] T_m in kelvin, ΔH_m in kcal/mol, and ΔC_p in kcal/mol/K units

3.4.2. Chemical denaturation studies:

The chemical denaturation of the proteins at five different temperatures, 293, 298, 303, 308 & 313 K was carried out using Gdm as a denaturant. The thermodynamic stability of the proteins in the presence of Arg, Lys, and Asp were evaluated and compared with the effect of Glu. Both the proteins followed two-state unfolding transitions in the presence of Arg, Lys, and Asp at all the five temperature conditions when denatured with Gdm (Figure 3.6 and 3.9). All the transition curves were fitted using equation 2.3. The calculated free energies of unfolding (ΔG_{unf}) and C_m values for RNase A are presented in Figure 3.7. The ΔG_{unf} and C_m values of RNase A decreased on increasing the concentration of Arg and increased on increasing Lys and Asp concentrations. Nevertheless, in all the cases, ΔG_{unf} and C_m values decreased with increase in temperature. The slope of the transition region (m_g values) remained almost the same even at the higher concentrations of Arg while it slightly increased at the higher concentrations of Lys and Asp. However, the change in m_g values with increase in temperature was insignificant (Figure 3.8).

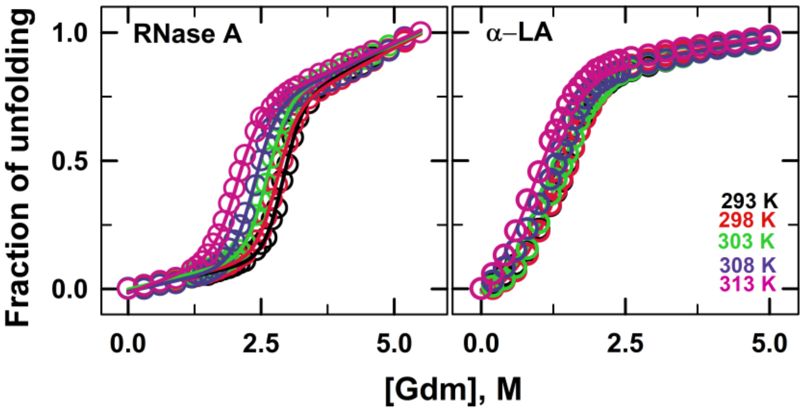

Figure 3.5. Gdm-induced unfolding of RNase A and α-LA each measured at different temperatures ranging from 293 to 313 K. The data is reproduced from Figures 2.11A and 2.14A of the previous chapter for comparison.

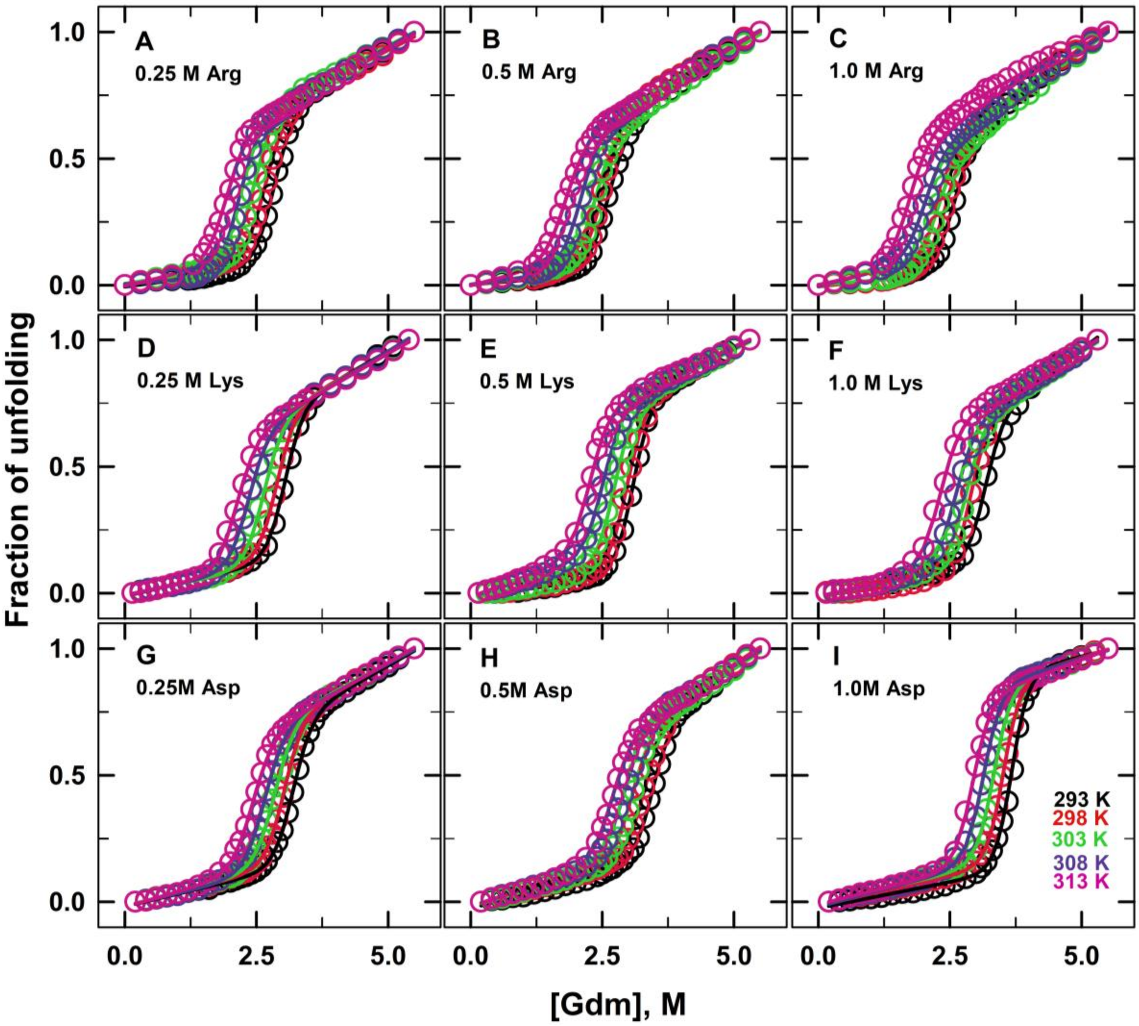

Figure 3.6. Gdm-induced unfolding of RNase A in the presence of varying concentrations (0.25, 0.5 and 1.0 M) of Arg (A-C), Lys (D-F), and Asp (G-I), each measured at different temperatures ranging from 293 to 313 K at the interval of 5 K. The solid lines represent data-fit for two-state unfolding transition using equation 2.3.

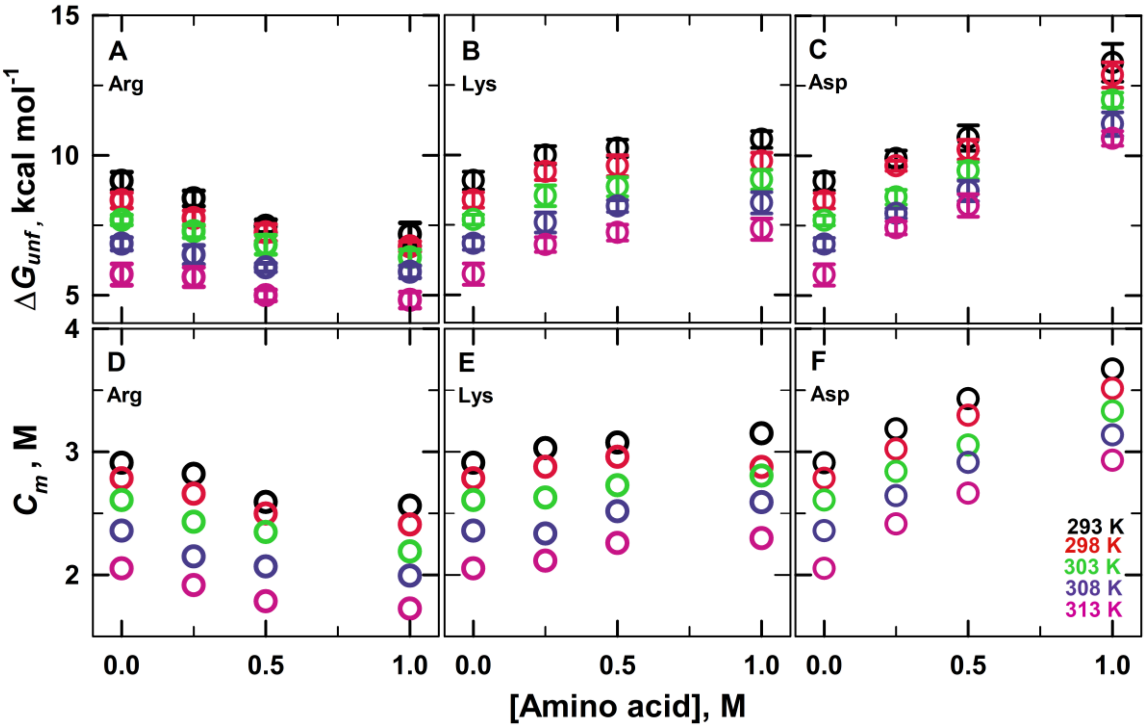

Figure 3.7. Change in free energy of unfolding (A-C) and C_m-values (D-F) of RNase A upon Gdm-induced unfolding in varying concentrations of Arg, Lys, and Asp each measured at different temperatures ranging from 293 to 313 K.

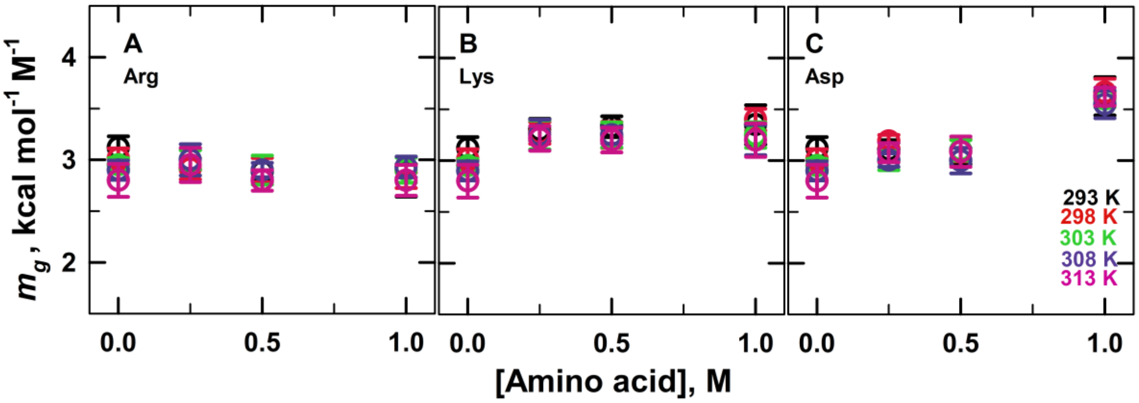

Figure 3.8. The slope of Gdm-induced chemical denaturation transitions (m_g-values) of RNase A in the presence of different concentrations of Arg, Lys, and Asp each measured between 293 K and 313 K at the interval of 5 K.

In the case of α-LA as well, the unfolding free energy and C_m values followed the similar trend as that observed in RNase A i.e., Arg decreased the free energy of unfolding whereas Lys and Asp increased (Figure 3.10). The m_g-values were not significantly affected by changing the concentration of Arg, Lys, and Asp (Figure 3.11).

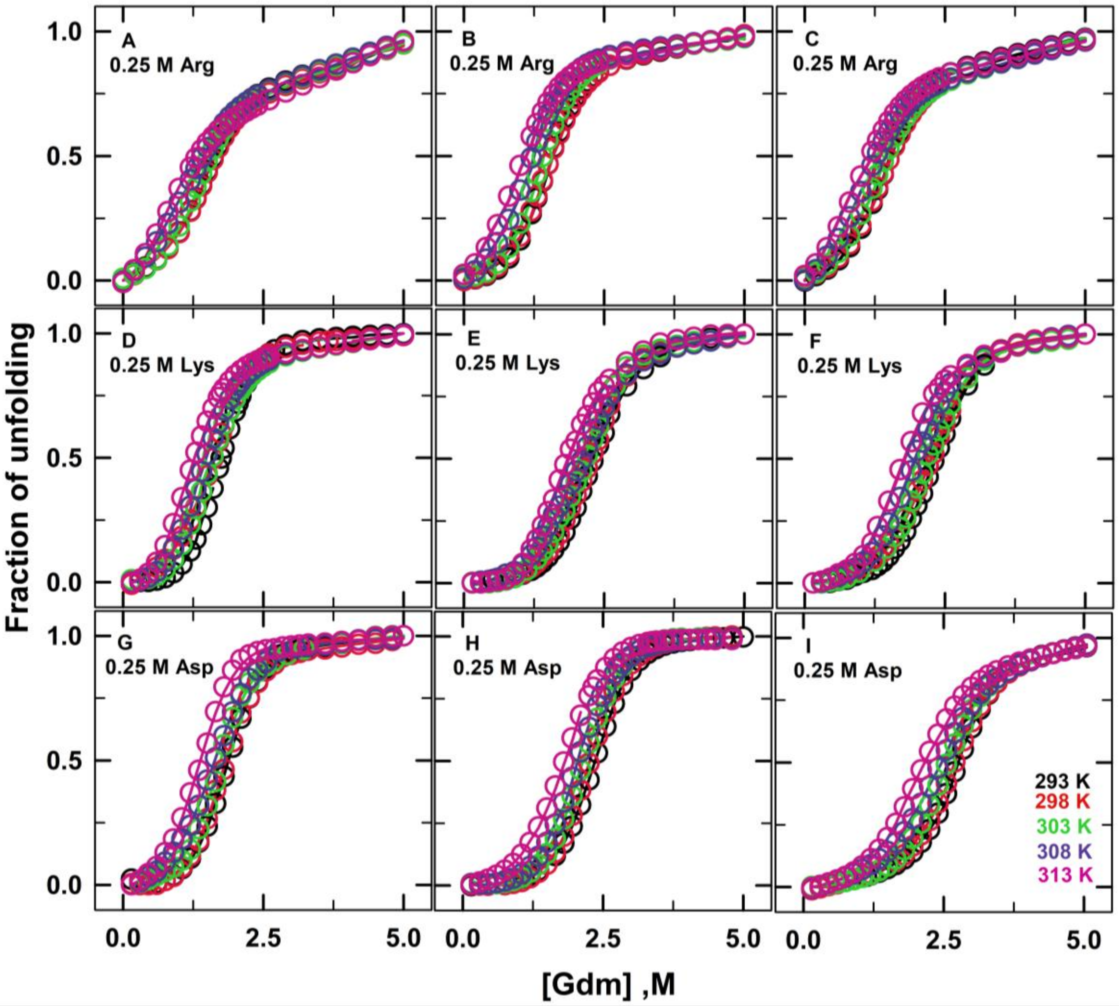

Figure 3.9. Gdm-induced unfolding of α-LA in the presence of varying concentrations (0.25, 0.5 and 1.0 M) of Arg (A-C), Lys (D-F), and Asp (G-I), each measured at different temperatures ranging from 293 to 313 K at the interval of 5 K. The solid lines represent data-fit for two-state unfolding transition using equation 2.3.

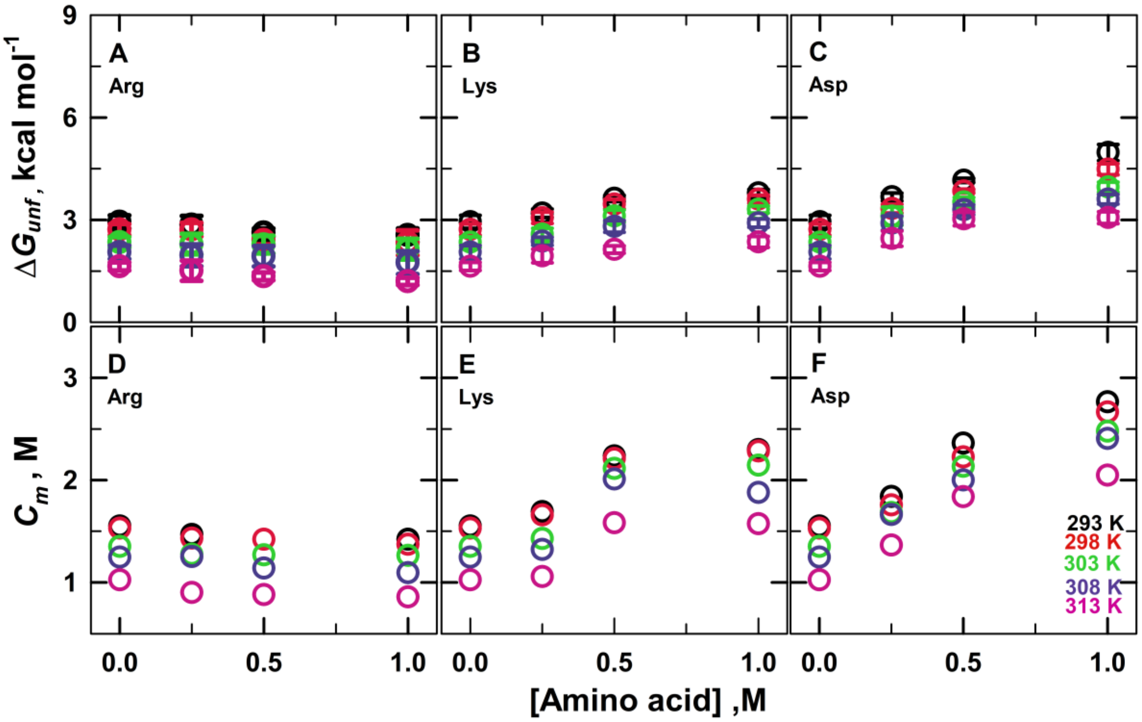

Figure 3.10. Change in free energy of unfolding (A-C) and C_m-values (D-F) of α-LA upon Gdm-induced unfolding in varying concentrations of Arg, Lys, and Asp each measured at different temperatures ranging from 293 to 313 K.

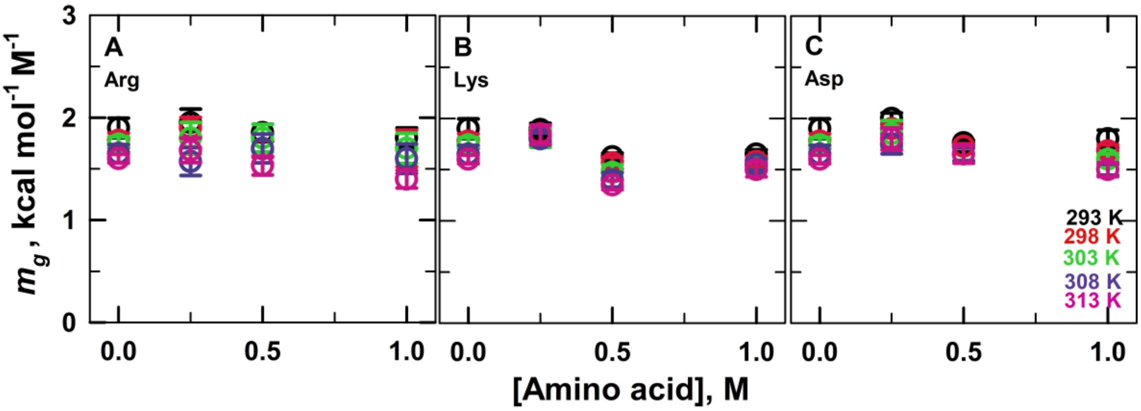

Figure 3.11. The slope of Gdm-induced chemical denaturation transitions (m_g-values) of α-LA in the presence of different concentrations of Arg, Lys, and Asp each measured between 293 K and 313 K at the interval of 5 K.

3.4.3. Structural changes in the presence of amino acids:

The tertiary structural changes in RNase A and α-LA in the presence of amino acids were monitored using near-UV circular dichroism (Figure 3.12). The CD spectra of both the proteins in the presence of Arg, Lys and Asp were not significantly altered.

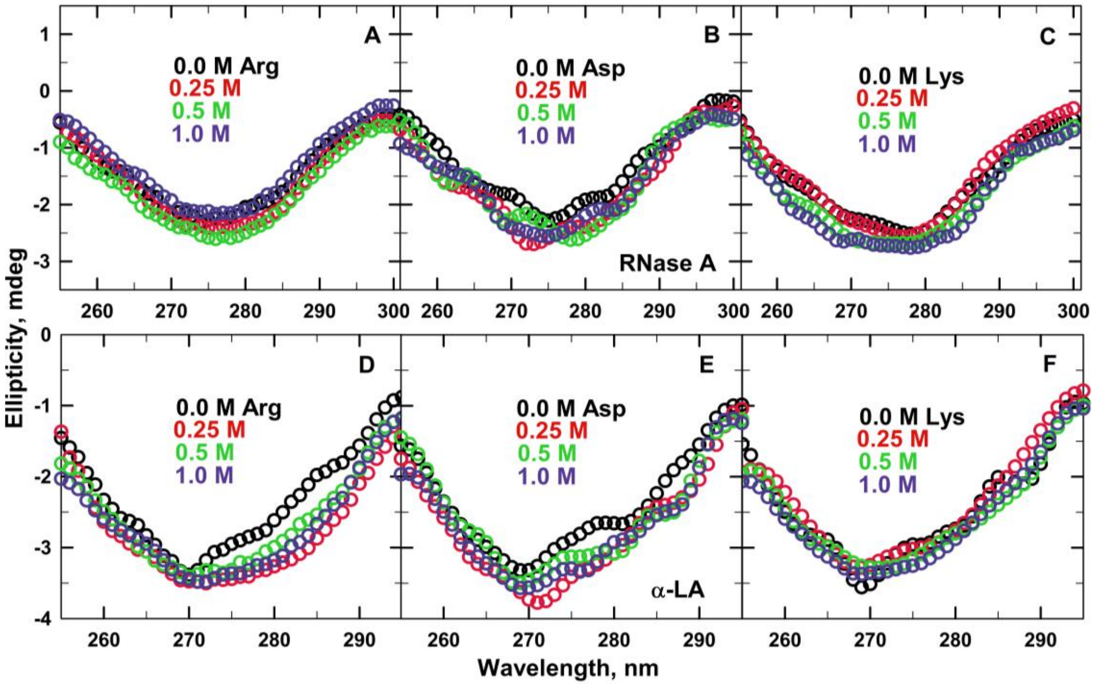

Figure 3.12. Near UV-CD spectra of RNase A (upper panels) and α-LA (lower panels) in the absence (black) and the presence of 0.25 (red), 0.5 (green), and 1.0 M (purple) of amino acid, (A and D) Arg, (B and E) Lys, and (C and F) Asp.

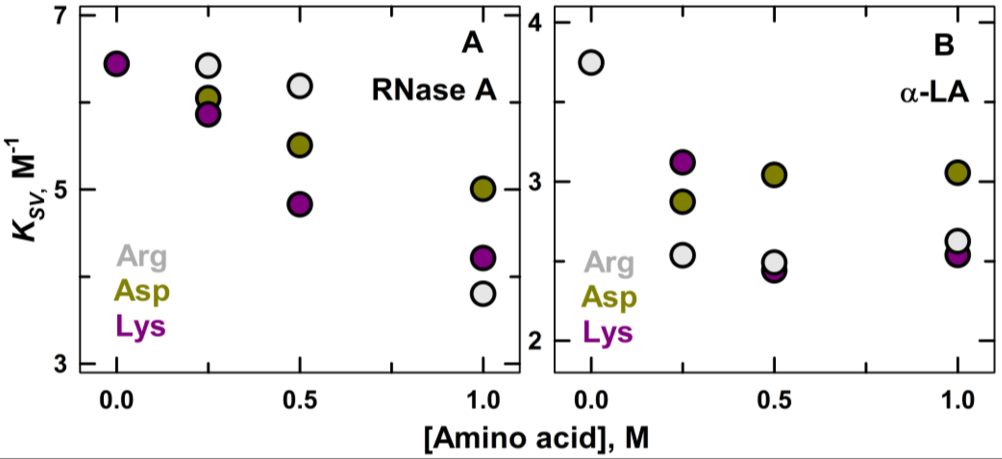

Figure 3.13. Stern-Volmer quenching constants (K_{SV}) measured for (A) RNase A and (B) α-LA in the presence of Arg (gray), Asp (green), and Lys (pink). In all the experiments acrylamide was used as a quencher.

For further investigation on the extent of solvent exposure of the buried residues, the ability of acrylamide to quench the fluorescence emission of both the proteins was examined in the presence of the amino acids. In the case of RNase A (Figure 3.13 A), the addition of Arg at above 0.5 M decreased the effective quenching of acrylamide which resulted in a decrease in K_{SV} values whereas the addition of Lys and Asp gradually decreased the K_{SV}

values upon increasing their concentrations. In the case of α-LA (Figure 3.13 B) as well, the K_{SV} value was decreased with the addition of the amino acids, however, increasing the concentration of amino acids above 0.25 M (or 0.5 M in the case of Lys) did not further alter the K_{SV} values significantly. Similar results were observed earlier in the case of Glu as well (section 2.4.4 and figure 2.17 A). Among all the amino acids, the extent of reduction in K_{SV} values was less in Asp for both the proteins.

3.4.4. Volumetric analysis:

The partial molar volume and compressibility of the proteins depends on the dynamics of intrinsic volume and hydration volume. The partial molar volumes (V^o) of both RNase A and α-LA were decreased as the concentration of the amino acid was increased in the solution (Figure 3.14A-C). The V^o values, in turn, increased on increase in temperature from 293 K to 313 K for all the studied amino acids. The adiabatic compressibilities (K_s) of the proteins were also decreased with increasing concentrations of the amino acids (Figure 3.14D-F). However, the K_s values of both the proteins decreased upon increasing the solution temperature and the extent of decrease was gradually reduced at the higher amino acid concentrations.

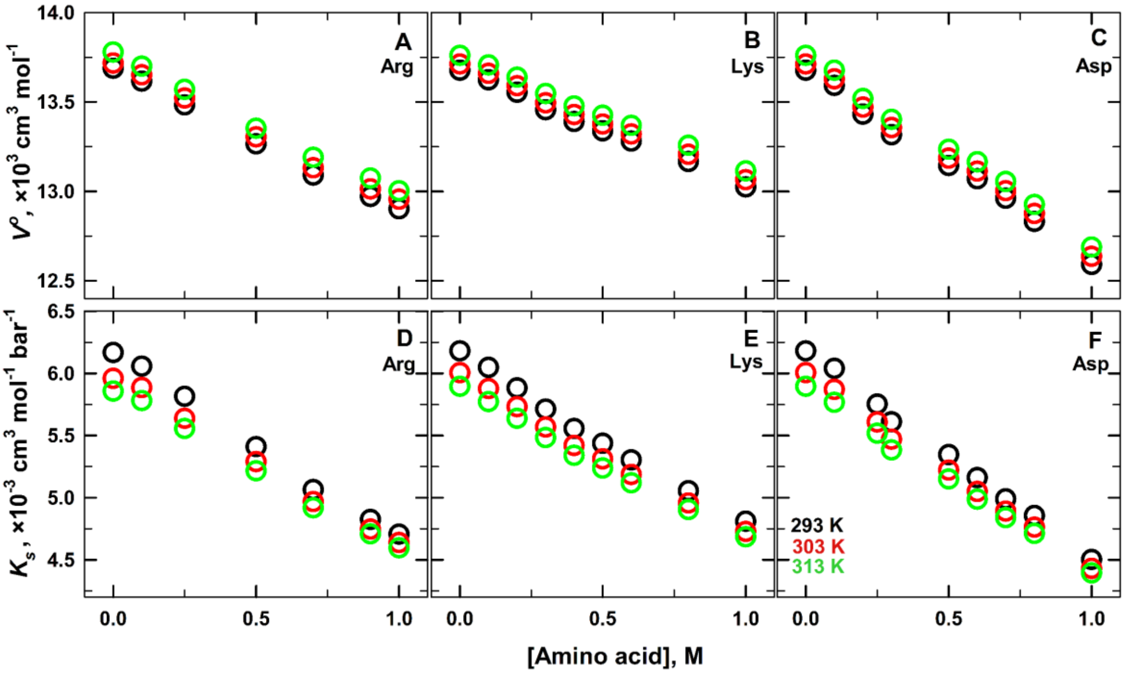

Figure 3.14. Partial molar volumes (upper panels) and adiabatic compressibilities (lower panels) of RNase A evaluated from density and acoustic measurements in different concentrations of amino acids, (A and D) Arg, (B and E) Lys and (C and F) Asp at 293 K (black circles), 303 K (red circles), and 313 K (green circles).

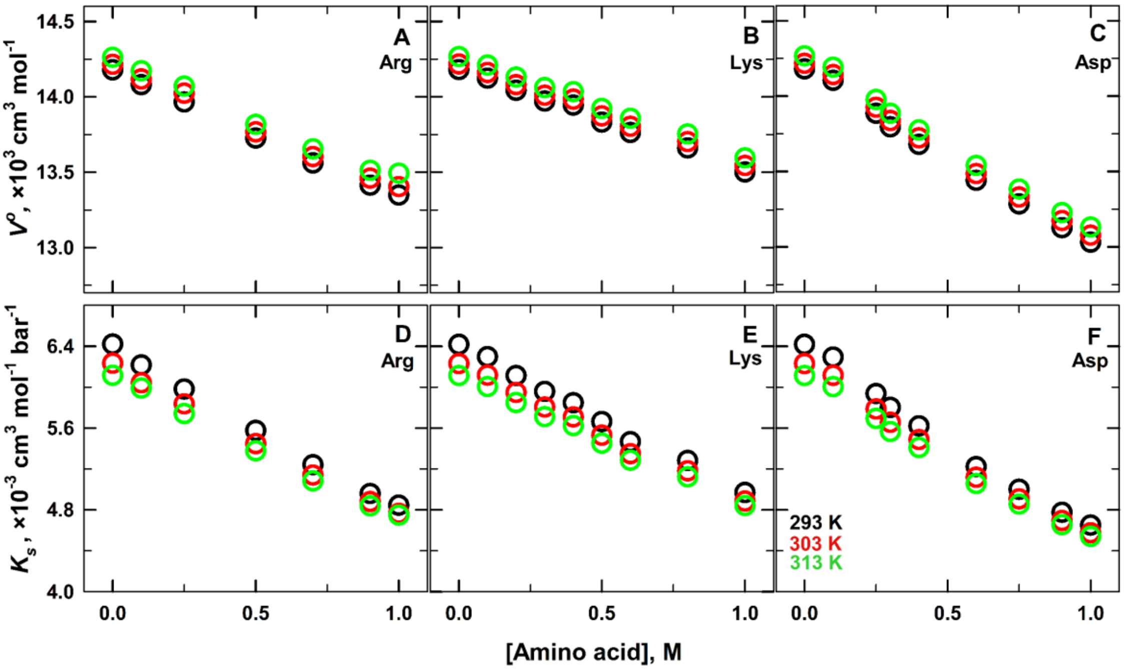

Figure 3.15. Partial molar volumes (upper panels) and adiabatic compressibilities (lower panels) of α-LA evaluated from density and acoustic measurements in different concentrations of amino acids, (A and D) Arg, (B and E) Lys and (C and F) Asp at 293 K (black circles), 303 K (red circles), and 313 K (green circles).

3.5. Discussion:

The free energy difference between the folded and unfolded conformations of proteins is less and the average among the globular proteins is roughly estimated to be ~11 kcal/mol suggesting a delicate balance of interaction energies in the proteins.[35] Nevertheless, retaining the native conformation is crucial for the function of proteins even at the stressed conditions. The biological systems commonly recruit osmolytes to meet this requirement[36-37] and amino acid class of osmolytes are one of the widely found molecules in different organisms.

3.5.1. Effect of charged-amino acids on heat-induced denaturation:

Arg is known for its "dual effect" on proteins. It reduces the aggregation of partially unfolded proteins and also destabilizes the native proteins.[18-19] This effect has been observed on α-LA, RNase A and lysozyme at slightly different solution conditions.[18,33] The present study also indicates that Arg could destabilize the model proteins, RNase A and α-LA at pH 7 (Figure 3.1). Arg is shown to interact via charge-charge and cation-π interactions with the proteins, thus alters the local structure of the proteins around their aromatic residues.[38] Lys is shown to stabilize lysozyme and BSA through preferential hydration of the proteins.[27,39] RNase A and α-LA is also stabilized by the addition of Lys. Though Asp is reported to slightly destabilize some proteins,[31-32] RNase A and α-LA is stabilized by Asp (Figure 3.3).

In order to compare the energy differences upon (de)stabilization, the change in unfolding free energy ($\Delta\Delta G_T$) due to the addition of charged amino acid is calculated at the thermal denaturation midpoint of the respective proteins (338.2 K for RNase A and 329.2 K for α-LA) from their thermal denaturation profiles (Figure 3.16). Destabilization of RNase A by Arg is slightly more than that of α-LA. Among the stabilizing AAs, Lys shows a similar effect on both the proteins whereas Asp provides more stabilization to RNase A. It may be noted that Glu induced stabilization free energy for both the proteins is similar (section 2.5.1 and Figure 2.19).

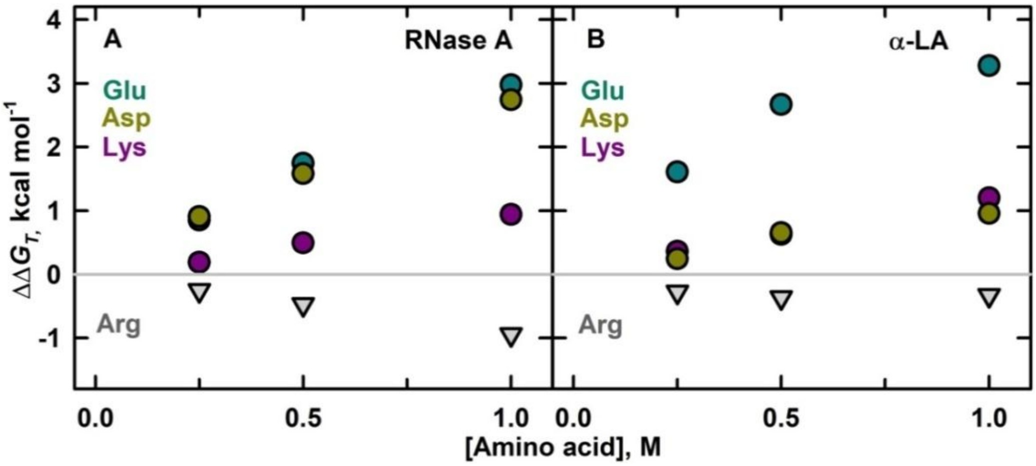

Figure 3.16. Change in the unfolding free energy of (A) RNase A and (B) α-LA measured at their transition midpoint, T_m (i.e., 338 K for RNase A and 329 K for α-LA) upon the addition of different concentrations Arg (gray), Glu (cyan), Asp (green), or Lys (pink). The solid gray line represents the $\Delta\Delta G_T$ in the absence of any amino acid.

3.5.2. Charged amino acids induce structural compaction:

Though the surface charge distribution on RNase A and α-LA are predominantly positive and negative, respectively, the stabilizing (or destabilizing) effect of the charged amino acids is similar on both the proteins. This suggests that the change in water structure in the protein hydration shell by the amino acids might be the major factor altering the stability rather than direct interactions. Further, the structural changes examined using near-UV CD and quenching experiments (Figures. 3.12 and 3.13) indicate that all the charged amino acids, irrespective of their effects on the protein stability, do not alter the tertiary contacts significantly and they, in fact, make the proteins slightly compact. This suggests that the stability changes are influenced by the difference in the interactions of amino acids with the denatured states of the proteins. The trivial changes observed in the heat capacity changes (ΔC_p) of the proteins during thermal denaturation process also support the fact that the extent of exposed hydrophobic residues in the presence of amino acids is similar to that of the native state. Earlier studies also proposed that Arg favourably interacts with the intermediates and unfolded conformational states.[19,38,40] Further, experiments on the effect of other stabilizing osmolytes such as polyols and sugars have shown that the osmolytes preferentially hydrate the native states more than the unfolded states of the proteins, thus, driving the N ↔ U equilibrium towards native state.[41-42] The stabilizing AAs also might follow the similar mechanism. Since the structural compaction for both RNase and α-LA is observed in the presence of these amino acids, the decrease in V^o and K_S values upon increasing the amino acids concentration can be ascribed to the decrease in the intrinsic volume of proteins rather than the decrease in its surface hydration. The net reduction of V^o and K_S upon addition of 1 M of Asp and Glu (from the previous chapter) are in comparable range and higher compared to Arg and Lys, thus, making them effective stabilizers. However, the V^o increases with increase in temperature suggesting an increase in its intrinsic volume due to structural relaxation at higher temperatures. At the same time, K_S values decrease with increase in temperature which can be due to the loss of intra-chain contacts and increase in surface hydration because of relaxed chain dynamics. The extent of reduction in K_S values with temperature decreases with increasing amino acid concentration re-emphasizing that at higher concentrations amino acids could counteract the chain relaxation induced by temperature increment.

3.5.3. Effect of charged amino acids on Gdm-induced unfolding:

Similar to the observations in heat-induced denaturation studies, Arg destabilizes, and Lys and Asp stabilize the proteins against Gdm-induced denaturation. All of the unfolding curves follow two-state transitions. However, the stabilization effect varies with temperature. Further, the extent of stability or destability attained by the protein in the presence of Arg, Lys, and Asp was calculated as the difference in unfolding free energy in the presence and the absence of respective amino acids, $\Delta\Delta G_{unf}$. In case of RNase A, among the stabilizing AAs, Asp stabilizes the protein more effectively at higher temperature than Lys and Glu (Figure 3.17). In α-LA, Asp shows lower stabilization effect compared to Glu and the extent of stability is slightly reduced at higher temperature (Figure 3.18). The extent of destabilization achieved by Arg is greater in RNase A than in α-LA.

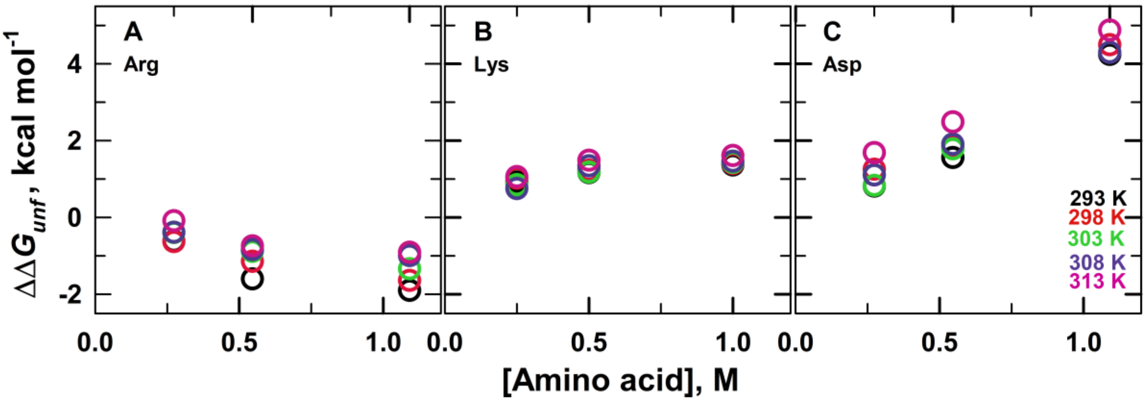

Figure 3.17. The extent of (de)stabilization of RNase A with the increasing concentrations of Arg (A), Lys (B), and Asp (C) calculated as the difference between the free energies of unfolding in the presence and the absence of respective amino acids measured at varying temperatures between 293 K and 313 K.

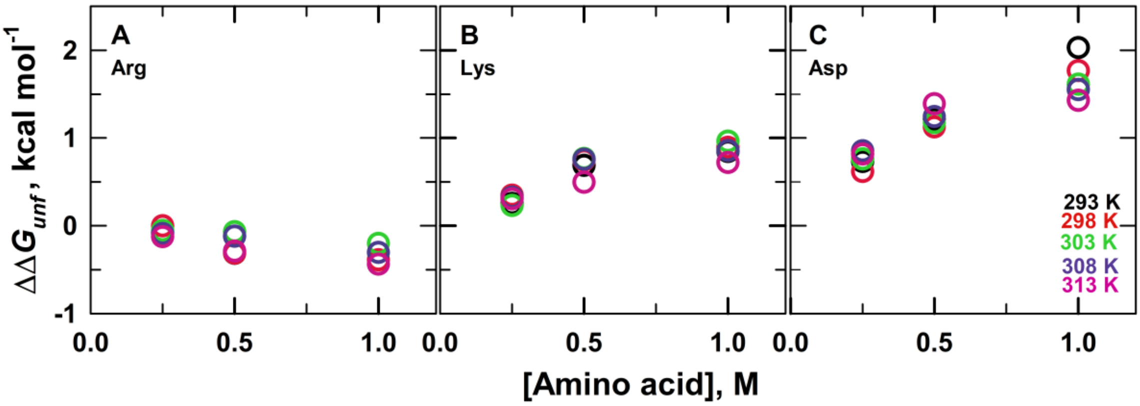

Figure 3.18. The extent of (de)stabilization of α-LA with the increasing concentrations of Arg (A), Lys (B), and Asp (C) calculated as the difference between the free energies of unfolding in the presence and the absence of respective amino acids measured at varying temperatures between 293 K and 313 K.

The m_g values of denaturation transitions increase in the presence of Lys and Asp in the case of RNase A while trivial changes are observed in α-LA. This variation of m_g values in both the proteins may be due to the extent of stabilization achieved by Lys and Asp is more in RNase A compared to α-LA. Since the m_g values represent the extent of denatured state exposed to the solvent, the above observations indicate that the denatured state of RNase A might become more solvent exposed upon the addition of Lys and Asp.[43] Neither the addition of Arg nor increasing the temperature significantly change the m_g values of both the proteins.

3.6. Summary:

The effect of charged amino acids on the thermodynamic stability of RNase A and α-LA is analyzed. Arg destabilizes the proteins whereas Asp and Lys stabilize the proteins. The order of stabilization for RNase A is found to be Arg < water < Lys < Glu ≤ Asp while for α-LA Arg < water < Lys ≤ Asp < Glu. All the charged amino acids does not affect the tertiary structure of the proteins; however, they induce structural compaction in the proteins. This could be attributed to the changes in the proteins' surface hydration and intrinsic volume by the addition of the amino acids. The results indicate that the charged amino acids might stabilize (or destabilize) the proteins through preferential hydration (or interaction), thus, show similar effects on the proteins with different surface charges.

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Chapter 4

Analysing Protein-Osmolyte Interactions by Molecular Dynamic Simulation

4.1. Abstract:

Amino acids are known to have profound effects as cosolvents on the thermodynamic stability of proteins and the inhibition of protein aggregation as well. However, the mechanistic insights into the molecular-level interactions are scarce. To illustrate the molecular mechanism of amino acid-induced (de)stabilization of proteins, the molecular dynamic (MD) simulations of two model proteins RNase A and α-LA were performed in the presence of four charged amino acids arginine (Arg), lysine (Lys), aspartate (Asp) and glutamate (Glu). As Arg has the side chain similar to that of guanidinium (Gdm), a chemical denaturant, and the experimental reports presented in the earlier chapter suggested that Arg destabilizes the proteins, MD simulations of the proteins were carried out in the presence of Gdm as well. RMSD and SASA derived from the simulations suggest that no major conformational changes are observed in the proteins during the simulations in the presence of any of the cosolvents. The density distribution functions and hydration fraction analysis reveals that the preferential interaction of the proteins with water increases upon the addition of amino acids; however, the extent of increase varies among the cosolvents. Among all the cosolvents, destabilizing cosolvents (Arg and Gdm) exhibit higher interaction compared to stabilizing amino acids (stAAs-Lys, Asp and Glu). The extent of interaction of amino acids with the proteins and the hydrational changes induced on the protein surface differ among the amino acids and the net outcome of these two effects might determine the stabilizing or destabilizing nature of the amino acid. Further, we quantified the interactions of these cosolvents with proteins at the residue-level and presented the results as polar (protein-side chain with cosolvent-side chain) and non-residue specific (includes the rest) interactions which is found to be specific to the cosolvents. The inter-molecular interaction energies evaluated from the simulations show that the net protein-water interaction energies are higher than the net protein-cosolvent interaction energies. Moreover, the interaction sites and energies for Arg and Gdm are similar which could be attributed to the common guanidinium group in their side chains. All these observations suggest that the preferential hydration or interaction could be the plausible mechanism of amino acid-induced protein stabilization or destabilization, respectively.

4.2. Introduction:

Proteins maintain a delicate equilibrium between the folded and unfolded ensembles and the folded conformations are generally favoured in their cellular environment. Any fluctuation in the dynamics of conformational this equilibrium might drastically alter the properties of the proteins.[1-3] Addition of osmolyte molecules might drift the equilibrium (N ↔ U) to either of the side depending on their interaction with the proteins. Although different theories on the plausible mechanism of interactions between proteins and osmolytes are proposed, complete molecular-level interactions couldn't be deduced from the available experimental data alone.[4-7] This urges the researchers to adapt computational methods for investigating the protein-cosolvent interactions. The structural dynamics of proteins, folding pathways, enzymatic reactions, and ligand binding is being investigated using molecular dynamic (MD) simulation methods for the past many years.[8-13] Recent advances in the MD simulation resources and the methodologies provide a greater opportunity to probe the protein dynamics at the atomic-level by sufficiently increasing the conformational sampling of the ensembles under desired physio-chemical conditions.[14-20] Dynamic simulations of the proteins in the presence of various cosolvents are also recently carried out to decipher the interacting mechanisms.[21-26]

Most of the reported MD simulation studies on protein-osmolyte interactions are mainly focussed on methylamine,[27-31] sugar,[23, 32-33] and polyol[21-22, 34] classes leaving the amino acid class of osmolytes largely unexplored. For instance, the MD simulation of lysozyme in the presence of sugars, such as maltose, sucrose, and trehalose, have shown that the sugars slowed down the dynamics of lysozyme due to the change in solvent dynamics (sugar-water interactions) rather than the protein-sugar interactions.[35-36] Among the three sugars, trehalose is the most efficient at stabilizing the protein by re-ordering the water's tetrahedral structure and forming clusters by self-association which in turn entraps the water molecules.[37-38] From an extensive analysis of the dynamics of chymotrypsin inhibitor 2 in the presence of different polyols, it is proposed that the capacity of the polyols to protect the proteins against thermal unfolding increases with an increase in the molecular volume and the fractional polar surface area of polyols.[21] The stabilizing nature of polyols stems from the indirect interactions that the preferential exclusion of polyols from the surface of the protein enhances the ordering of water structure, thus decreasing the water entropy.[21-22, 39] In contrast to the above observations, polyols are also shown to interact directly with peptides and help in stabilizing the α-helical structure of the peptides.[34]

Though there are extensive studies using various experimental and computational methods to understand the interactions between protein and denaturants or non-compatible osmolytes, the exact molecular mechanism of unfolding is still an on-going debate.[40-48] Of the two proposed mechanisms (direct and indirect), the direct mechanism is gaining significance with recent experimental evidences.[44-45,49] The direct mechanism hypotheses that urea interacts either with the protein backbone or the side chains or both through hydrogen bonding or electrostatic interactions whereas indirect mechanism hypotheses that urea's perturbing effects on the water structure leads to the exposure of hydrophobic groups to the solvent.[48,50] However, a few reports propose that the unified mechanism could explain the changes rather than the exclusive contribution of either of the mechanisms.[40,45-46] For instance, the contact coefficient of urea with amino acid side chains and the backbones evaluated from the MD simulation of urea in the presence of all the individual amino acids suggests that the aromatic and apolar side chains have the highest contact coefficients. This is followed by the backbone atoms, and polar and positively charged side chains while the negatively charged Asp and Glu expels urea from the vicinity of their side chains. These protein-urea contacts might prompt preferential exclusion of water molecules from the surface of the proteins, thus, operating along with the direct interaction of denaturants to unfold the proteins.[51] Further, the mechanical unfolding of lysozyme in the presence of urea and betaine also reveal that urea uniformly interacts with the protein residues on the surface through direct and water-mediated interactions. However, betaine is found to act more like a ligand than a cosolvent due to its residue-specific interactions mainly with the basic amino acids.[25] Even the mechanism of counteracting interaction of TMAO, a compatible osmolyte, with the proteins is still elusive. A few studies propose that the presence of TMAO enhances the water structure[52] while the others argue that strong TMAO-urea interactions expel the urea away from the protein's vicinity.[28, 53-55] It is also proposed that TMAO competes with urea at the protein surface, thereby counter protecting the proteins.[4, 28, 54]

There are earlier attempts to examine the interactions of amino acid osmolytes with the protein residues. For instance, MD simulations of lysozyme performed in the presence of guanidine, arginine, and lysine suggest that the hydration layer is disturbed more in the presence of arginine than guanidine and lysine. In the presence of guanidine, the intra-molecular hydrogen bonds (side chain-side chain) are lost which might result in the unfolding of the protein. All the three additives show higher contact coefficients for acidic followed by polar and aromatic residues. However, guanidine interacts more preferentially with the protein residues compared to the guanidinium side chain of arginine which is attributed to the steric hindrance of Arg.[56] Also, it is suggested that the zwitterionic nature of Arg leads to the self-assembly of arginine molecules.[56-57] Another study shows that arginine's property of inhibiting the protein aggregation can be attributed to its cation-π interactions and salt-bridge formation with the aromatic and charged residues of the proteins, respectively.[57] Further, the MD simulation of α-lactalbumin in the presence of arginine shows that guanidinium group of arginine specifically interacts with the acidic amino acids and their amides (Gln, Asn).[58] Another osmoprotectant, proline, is also found to be preferentially excluded from the protein surface and form molecular aggregates thereby resulting in enhanced water-water interactions driving the protein stability.[26]

Despite the use of many additives to stabilize the proteins during protein purification, therapeutic formulations, and many biotechnological applications[59-60] the molecular-level interactions of protein-osmolytes is yet to be fully established. This chapter provides some insights into the protein-charged amino acids and protein-ionic denaturant interactions by employing computational methods. The MD simulations of ribonuclease A (RNase A) and α-lactalbumin (α-LA) in the presence of four charged amino acids arginine (Arg), lysine (Lys), aspartic acid(Asp) and glutamic acid (Glu) were carried out. In addition, the effect of guanidinium (Gdm) was also examined and compared with the effects of arginine on the proteins. Apart from the global properties such as mean residue fluctuations, the fraction of solvent molecules around the protein, the fraction of intermolecular hydrogen bonds, and the contribution of electrostatic and van der Waals interaction energies were evaluated. Further residue specific interactions of the osmolytes were also analysed. The overall analysis suggests that it is the extent of preferential hydration or interaction of the amino acids with the proteins that determine the stabilizing or destabilizing property of the amino acid cosolvents. As all the amino acids exhibit both preferential hydration and interactions with the protein moieties, the net outcome of these two factors would, therefore, decide their compatible or non-compatible nature.

4.3. Methods:

4.3.1. Molecular dynamic (MD) simulation studies:

The crystal structures of RNase A (PDB id: 7RSA) and α-LA (PDB id: 1HFX) were obtained from the Protein Data Bank (www.rcsb.org). After removing the co-crystallized ligands, the proteins were simulated in the absence and the presence of amino acids, Arg, Lys, Asp, and Glu. In addition to this, simulations using Gdm as a cosolvent were also performed. The number of cosolvent molecules added was such that the final concentration of the cosolvent in the system was nearly 0.5 or 1.0 M (Table 4.1). All of the simulations were carried out in Gromacs 4.6.3[61] using the CHARMM27 force field[62] and TIP3P water model.[63] The parameters for amino acids were obtained from the same force field while the Gdm parameters were assigned with the help of CHARMM General Force Field (CGenFF) program.[64-65] Initially, the systems were energy minimized with steepest descent method and equilibrated under NVT followed by NPT conditions at 300 K and 1 atm for 200 ps each. The production simulations were carried out for 50 ns. A cut-off of 1.0 nm was used for short-range Coulombic and Lennard–Jones (LJ) interactions. Long-range electrostatic interactions were treated by Particle mesh Ewald (PME) scheme.[66] LINCS algorithm was employed to constrain all the bonds.[67] The coordinates and energies were collected at every 10 ps interval. The periodic boundary condition was applied to exclude the edge effects. The last 20 ns of the production runs were considered for further analysis.

Table 4.1: Details of the number of cosolvents molecules in MD simulations

[cosolvent], M	**Number of cosolvents**				
	Arg	**Lys**	**Asp**	**Glu**	**Gdm**
RNase A					
0.5	18	18	18	18	18
1.0	34	37	37	36	36
α-LA					
0.5	18	17	17	18	18
1.0	34	32	32	36	36

4.3.2. Analysis:

The simulation trajectories were analysed using various tools available in Gromacs 4.6.3. The g_rms program was used to compute the root-mean-square deviation of Cα atoms of the proteins. The solvent accessible surface area (SASA) for both the proteins over the course of simulation was calculated by g_sas program with a probe radius of 0.14 nm. Rest of the analyses were carried using the last 20 ns of the simulation trajectories. The g_rdf tool was used to extract the radial distribution functions (RDFs) between the heavy atoms of protein and solvent molecules. The number of waters and amino acids within the first and second hydration shells were obtained using g_select program and redundancy was removed using an in-house R-code. In addition, g_hbond was used to calculate the number of hydrogen bonds (H-bonds) between protein and solvent molecules.

4.3.2.a. Hydration fraction (χ_{hyd}) of protein:

The number of water molecules and the amino acids in the first and second hydration shells of the proteins was calculated at each time-step during the last 20 ns of MD simulation. The obtained numbers were averaged over the number of frames and used for further calculations. The variation in the distribution of water and amino acids around the proteins at different conditions was evaluated as a ratio between the fraction of water molecules and the fraction of amino acid found on the surface of the protein. This is a slightly modified form of "local-bulk partition coefficient K_p" which represents the affinity of cosolvent molecules to the solute.[31] This modified parameter could represent the hydration fraction (χ_{hyd}) of the protein surface within a given distance (r),

$$\chi_{\mathrm{hyd}} = \frac{(n_{\mathrm{W}}/N_{\mathrm{W}})}{(n_{\mathrm{AA}}/N_{\mathrm{AA}})} \qquad (1)$$

where n_{w} and n_{AA} represent the number of water and amino acid molecules around the surface of the protein within the distance r and N_{w} and N_{AA} represent the total number of water and amino acid molecules in the system. Hydration fractions were separately calculated for the first (r = 0.32nm) and second (r = 0.44nm) hydration shells of the proteins.

4.3.2.b. Hydrogen bond analysis:

The number of hydrogen bonds between the protein and solvents was calculated with a distance cut-off of 0.35 nm and an angle cut-off of 30° between donor and acceptor-hydrogen as a geometric criterion. The averaged value of extracted H-bonds over the frames corresponding to the last 20 ns was considered for further analysis. The mean fraction of hydrogen bonds formed between protein-water ($\text{Ƒ}_{P\text{-}W}$), protein-amino acid ($\text{Ƒ}_{P\text{-}AA}$), and protein-Gdm ($\text{Ƒ}_{P\text{-}Gdm}$) in the system during the simulation was evaluated as follows:

$$\text{Ƒ}_{P-W} = \frac{\text{protein}-\text{water H}-\text{bonds}}{(\text{protein}-\text{water H}-\text{bonds} + \text{protein}-\text{AA H}-\text{bonds})} \qquad (2)$$

$$\text{Ƒ}_{P-AA} = \frac{\text{protein}-\text{AA H}-\text{bonds}}{(\text{protein}-\text{water H}-\text{bonds} + \text{protein}-\text{AA H}-\text{bonds})} \qquad (3)$$

The fractions of $f_{P\text{-}W}$ and $f_{P\text{-}Gdm}$ for the systems containing Gdm as cosolvent were also evaluated in a similar way.

4.3.2.c. Residue-level interactions:

The surface amino acid residues of the proteins which are interacting with the added cosolvent were identified using a distance cut-off of 0.5 nm for last 20 ns. The obtained interaction counts were averaged over the number of frames. This value for each residue was further divided by the total number of corresponding residue available in the protein. The interactions were then grouped into backbone and side chain interactions, since the backbone interactions could be common among the proteins whereas the side chain interactions are residue-specific. The interaction energies for protein-water and protein-cosolvent pairs in the systems were analysed in GROMACS by following the method described by Lindgren *et al*.[68] The energies were further separated into Coulombic and van der Waals interaction contributions.

4.4. Results:

4.4.1. Global Analysis:

The root mean square deviation (RMSD) of the atomic positions with respect to the initial structure of the protein provides a comprehensive measure of conformational changes in the protein during the simulation. The positional changes in Cα atoms of the residues can be considered as a representative measure of fluctuations occurring on the individual amino acids in the protein. The RMSD of Cα atoms calculated for both RNase A (Figure 4.1 A-D) and α-LA (Figure 4.2 A-D) did not show significant changes upon the addition of any of the amino acids. The average values of RMSD of the proteins were 0.14 ± 0.02 nm and 0.15 ± 0.02 nm for RNase A and α-LA, respectively. In the presence of the cosolvents, the average values were found to be around 0.11-0.17 nm for RNase A and 0.14-0.18 ± 0.02 nm for α-LA. Also, the total solvent accessible surface area (SASA) of the proteins was analysed which largely remained unaltered for both the proteins on the addition of amino acids (Figures 4.1E-H and 4.2E-H). For further insight, the total SASA was bifurcated into hydrophobic and hydrophilic surface areas (Figures 4.3 and 4.4). The hydrophobic surface area was found to be increased for both the proteins with the addition of any of the amino acids whereas the hydrophilic surface area was decreased. However, no significant change in SASA was observed during the course of the simulations against time.

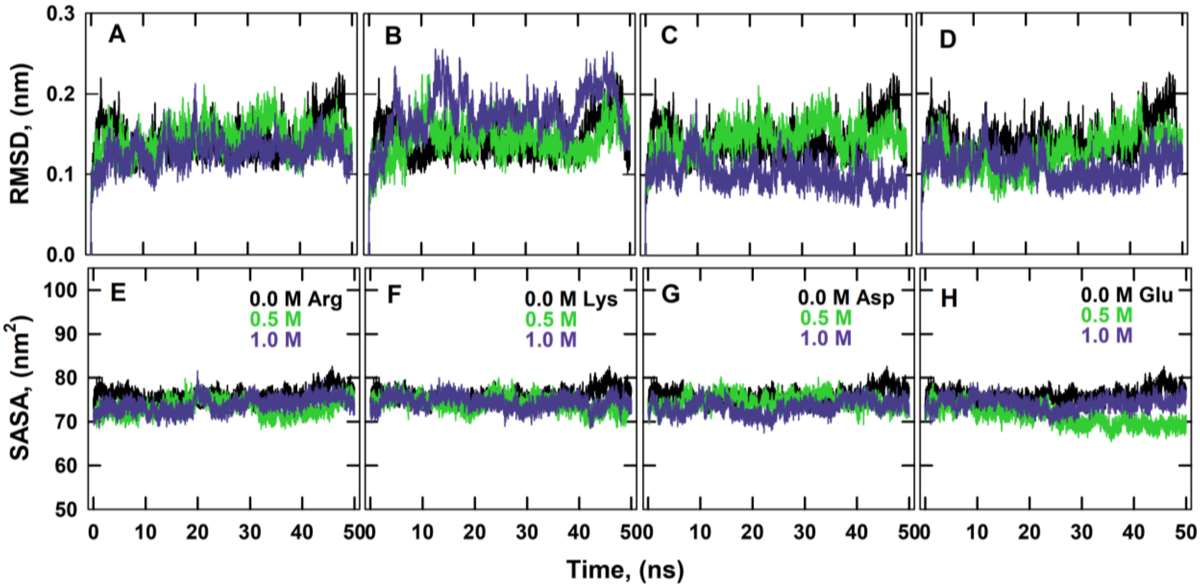

Figure 4.1. The change in RMSD (upper panels) and the total solvent accessible surface area (lower panels) in RNase A during the simulation of the protein in the absence (black) and in the presence of 0.5 M (green) and 1.0 M(purple) of the amino acids, Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

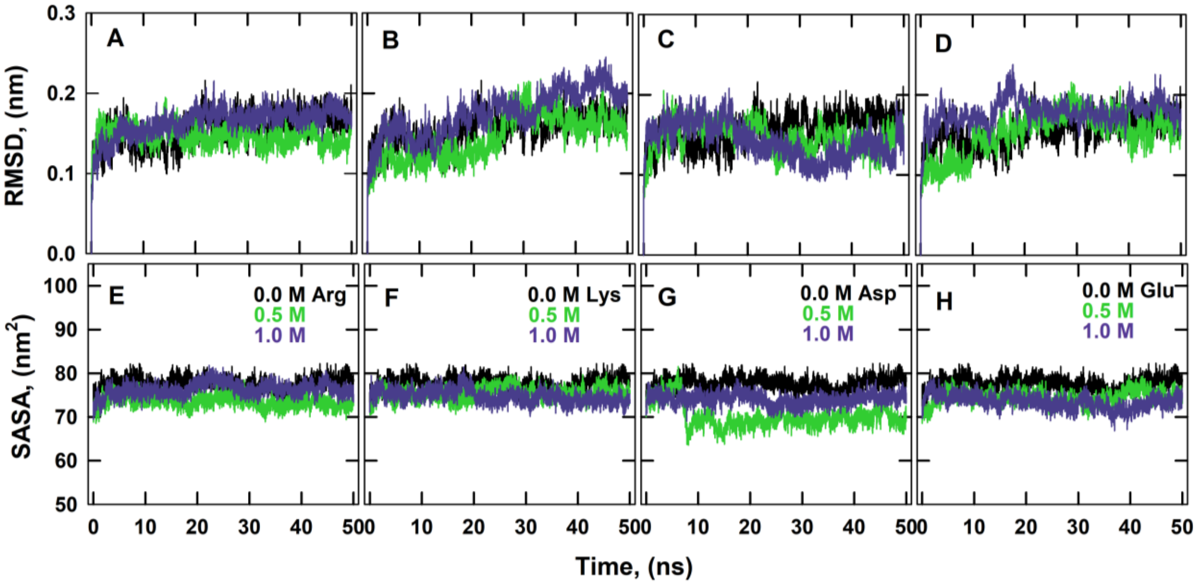

Figure 4.2. The change in RMSD (upper panels) and the total solvent accessible surface area (lower panels) in α-LA during the simulation of the protein in the absence (black) and in the presence of 0.5 M (green) and 1.0 M(purple) of amino acids, Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

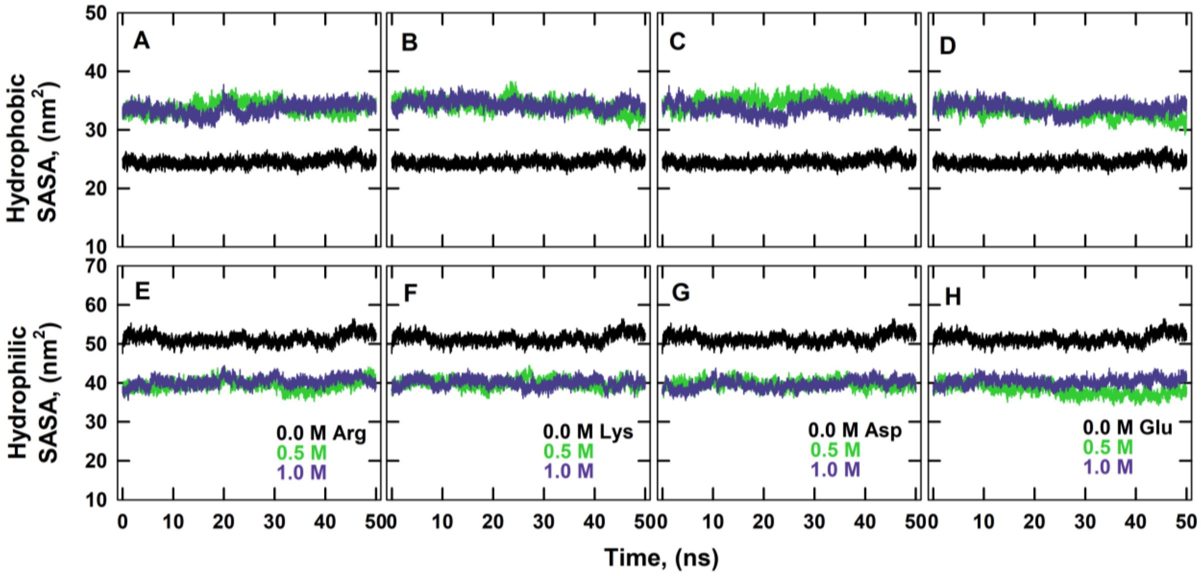

Figure 4.3. The total SASA of RNase A is divided into hydrophobic (upper panels) and hydrophilic (lower panels) SASA values. The values are calculated in the absence and the presence of Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

The addition of 0.5 or 1 M of Gdm also didn't show significant changes in the RMSD and total SASA of both the proteins (Figure 4.5). Although RMSD and total SASA remains unaltered with the progress of simulation time, the hydrophobic SASA was observed to be increased and hydrophilic SASA was decreased upon addition of Gdm similarly to the changes observed in the presence of amino acids.

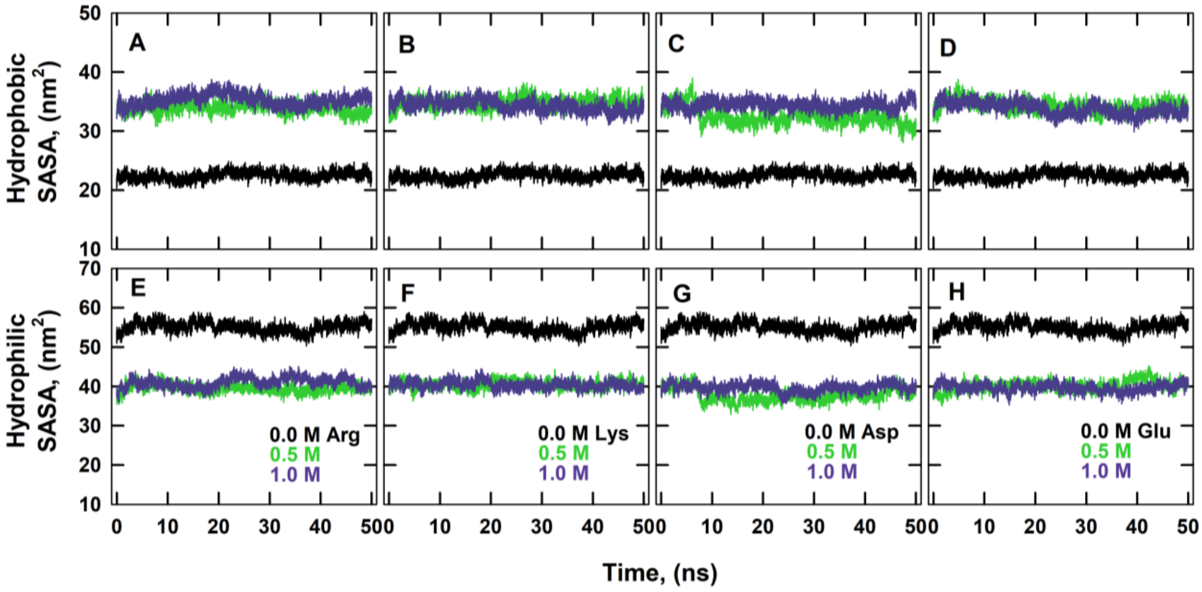

Figure 4.4. The total SASA of α-LA is divided into hydrophobic (upper panels) and hydrophilic (lower panels) SASA values. The values are calculated in the absence and the presence of Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

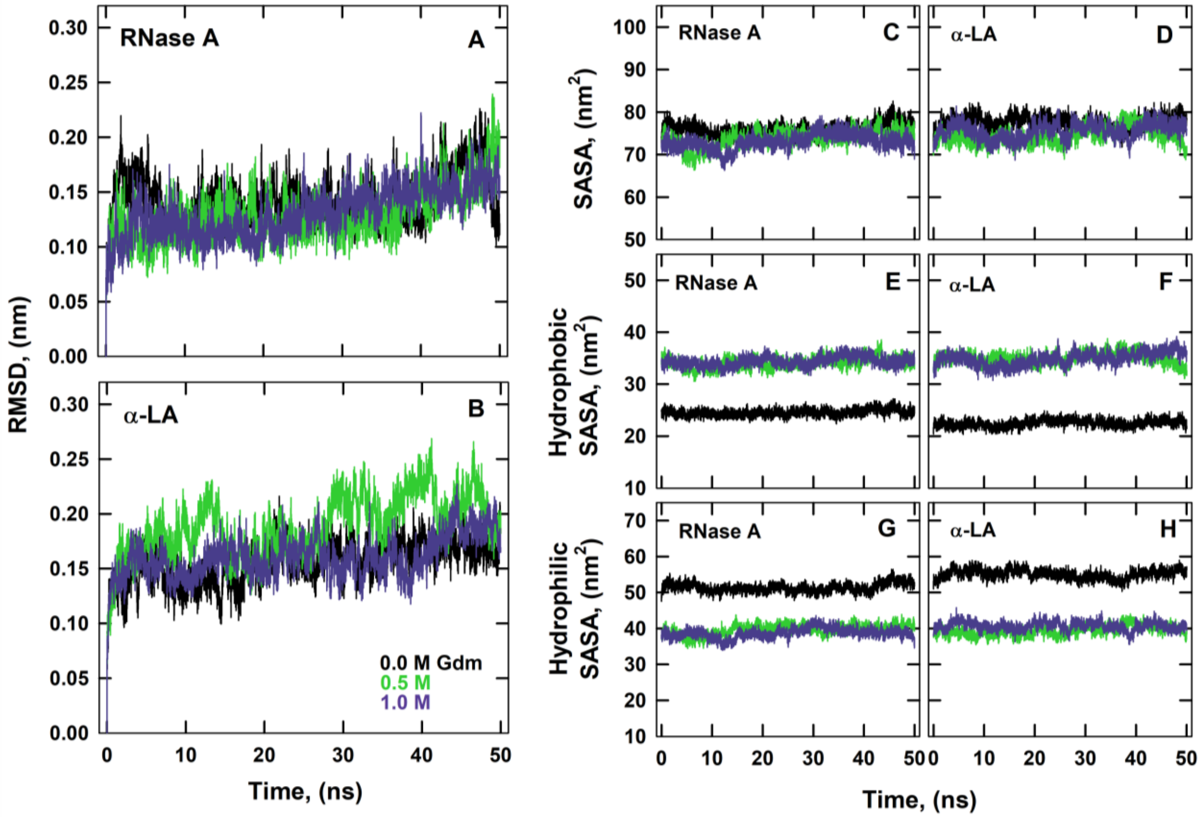

Figure 4.5. The change in RMSD (A & B), the total SASA (C & D), hydrophobic SASA (E & F) and hydrophilic SASA (G & H) during the simulation of RNase A and α-LA in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of Gdm.

4.4.2. Radial distribution functions:

The radial distribution function (RDF) measured around the protein molecule provides the probability density of water and cosolvent molecules against the distance from the surface atoms of the protein. The changes in RDFs would help to understand the variation in the solvation layer of the proteins induced by the amino acids (Figures 4.6 and 4.7). The RDF of water around the protein in the absence of any cosolvent showed the first maxima at 0.28 nm which is generally attributed to the tetrahedrally oriented water molecules around the protein heavy atoms.[21, 52] The consecutive small peaks found at 0.38 and 0.47 nm could represent second and third solvation shells. Addition of amino acids did not alter the positions of these peaks; however, the height of the peaks increased with increase in the concentration of amino acids suggesting an increase in water density around the proteins in the presence of the amino acids. Similarly, the RDFs of amino acids around the proteins were also analysed. As the concentration of the amino acid increased in the system, the density of the amino acid was reduced in first and second hydration shells of the proteins. This complemented with the observed increase in water density.

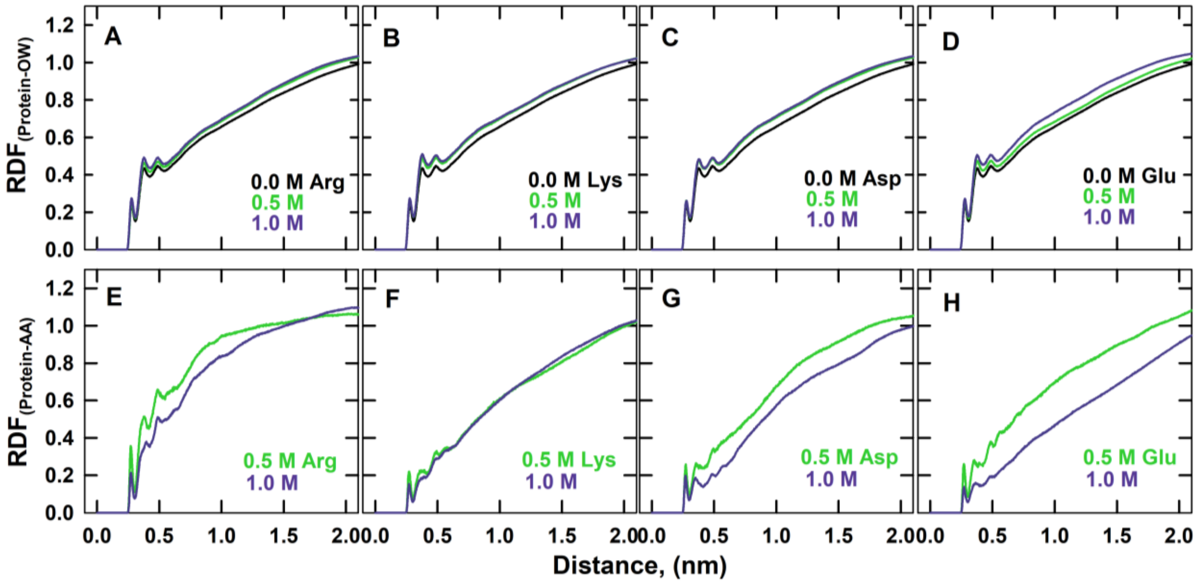

Figure 4.6. Radial distribution function of water (upper panels) and amino acids (lower panels) around RNase A in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of amino acids, Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

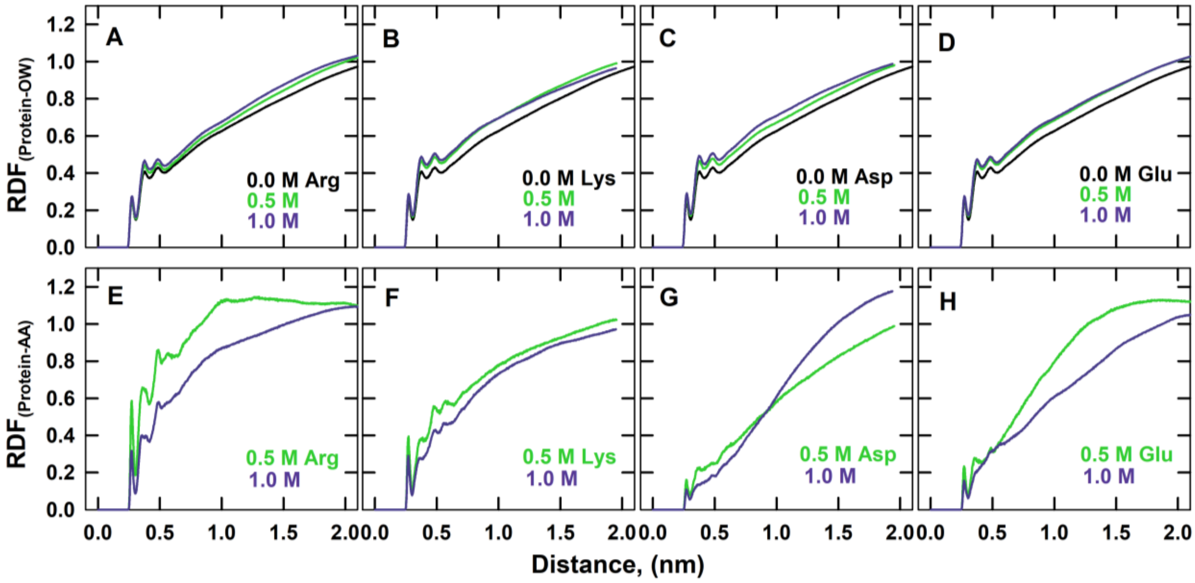

Figure 4.7. Radial distribution function of water (upper panels) and amino acids (lower panels) around α-LA in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of amino acids, Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

Among all the amino acids, the density of Arg was relatively larger than the other amino acids suggesting a higher affinity of the proteins towards Arg. Addition of Gdm, an ionic denaturant, slightly increased the density of water around the proteins and the density of Gdm slightly decreased with increase in its concentration (Figure 4.8). Nevertheless, the height of the peaks which represents the probability of Gdm occurrence near to the protein was found to be higher than that of Arg and other amino acids as well.

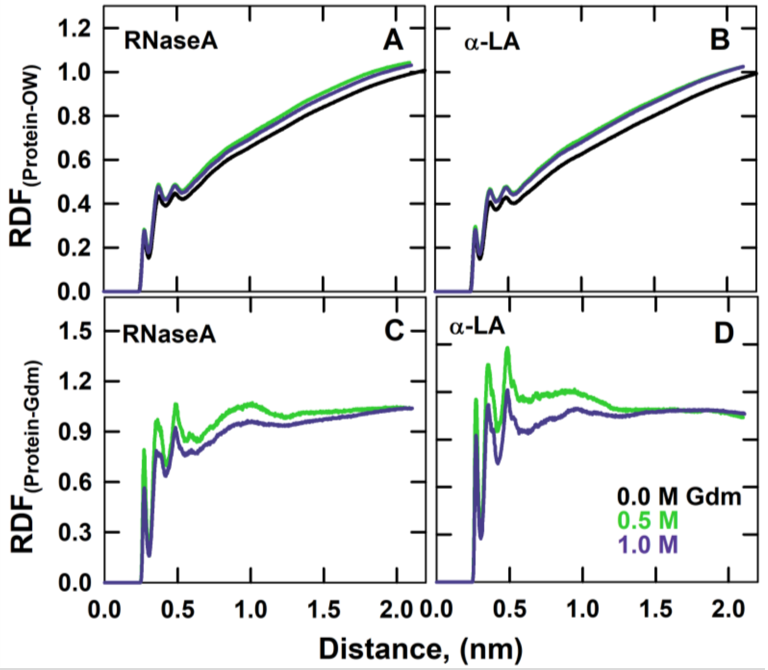

Figure 4.8. Radial distribution function of water (upper panels) and Gdm (lower panels) around RNase A (A & C) and α-LA (B & D) in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of Gdm.

4.4.3. Solvation properties:

To quantify the solvation effects, hydration fractions (χ_{hyd}) around the proteins were calculated in the absence and the presence of the amino acids (Figure 4.9 and 4.10). As the concentration of the amino acid was increased in the system, the fraction of water found around the first and second hydration shells of the proteins increased. Asp and Glu showed a larger increase than Lys which was higher than Arg. The extent of hydration was slightly more for RNase A compared to α-LA in the presence of any of the amino acids. For further analysis on the surface hydration of the proteins, the number of protein-water and protein-amino acid hydrogen bonds was evaluated at all the conditions (Figure 4.11 and 4.12). The results evidently showed that the fractions of protein-water H-bonds were marginally reduced in both RNase A and α-LA, while the fraction of protein-amino acid H-bonds increased with increase in the concentration of the amino acids. In all the cases, the protein surface was dominant with a higher fraction of protein-water hydrogen bonds (>0.8) over the protein-amino acid hydrogen bonds (<0.15).

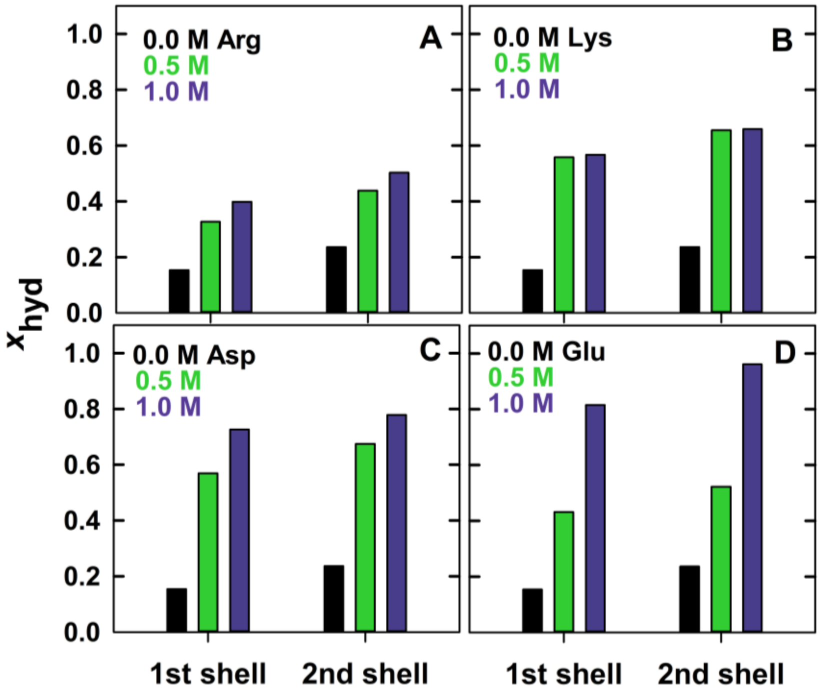

Figure 4.9. Hydration fraction around RNase A calculated at the first and second hydration shells of the protein in the absence (black) and in the presence of 0.5 M (green) and 1.0 M(purple) of the amino acids, Arg (A), Lys (B), Asp (C) and Glu (D).

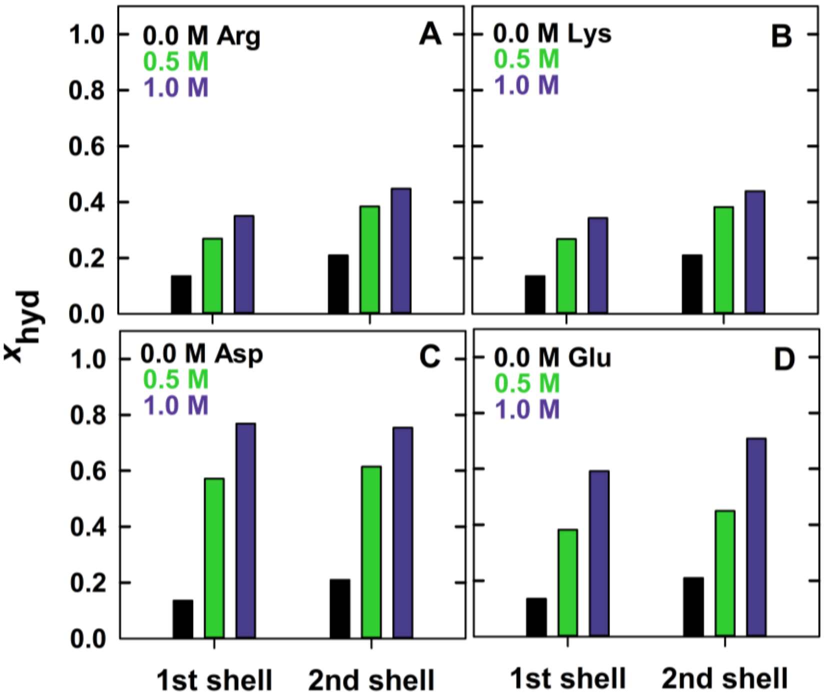

Figure 4.10. Hydration fraction around α-LA calculated at the first and second hydration shells of the protein in the absence (black) and in the presence of 0.5 M (green) and 1.0 M(purple) of the amino acids, Arg (A), Lys (B), Asp (C) and Glu (D).

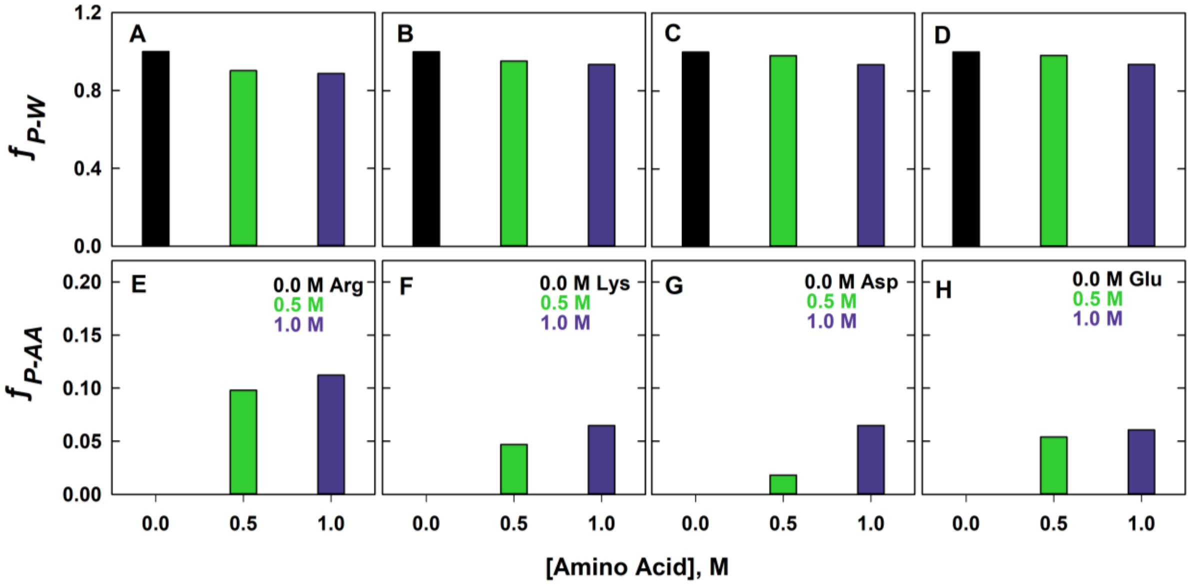

Figure 4.11. The fraction of intermolecular hydrogen bonds between RNase A and water (upper panels) and the fraction of intermolecular hydrogen bonds between RNase A and the amino acids (lower panels) calculated in the absence and in the presence of 0.5 M and 1.0 M of the amino acids, Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

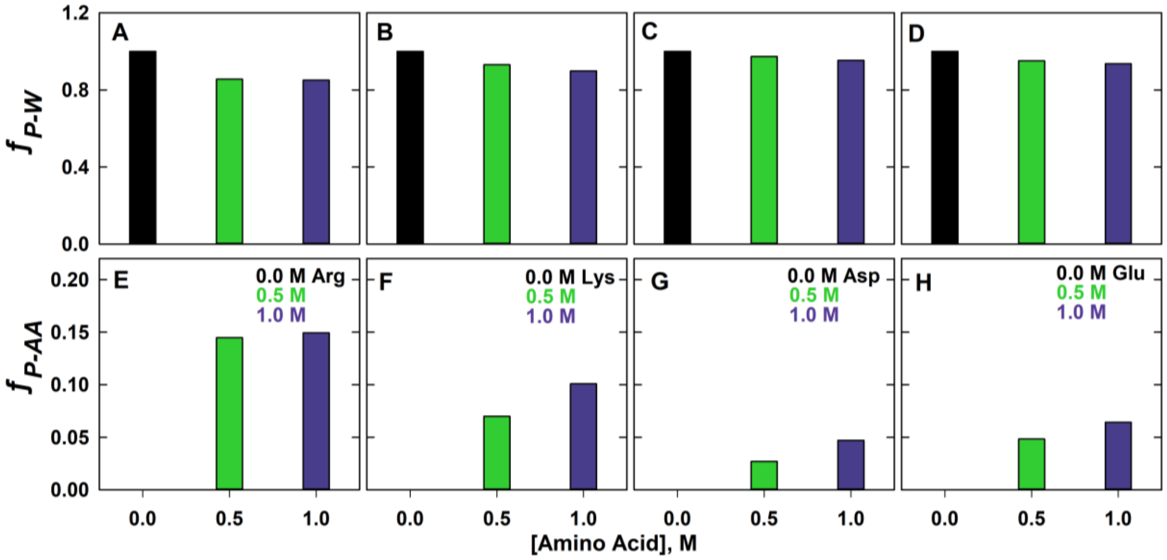

Figure 4.12. The fraction of intermolecular hydrogen bonds between α-LA and water (upper panels) and the fraction of intermolecular hydrogen bonds between RNase A and the amino acids (lower panels) calculated in the absence and in the presence of 0.5 M and 1.0 M of the amino acids, Arg (A & E), Lys (B & F), Asp (C & G) and Glu (D & H).

Similar results were observed in the presence of the denaturant, Gdm as well (Figure 4.13). The hydration fractions around the proteins were increased by the addition of Gdm into the system. As observed in the cases of amino acid cosolvents, the fraction of protein-water hydrogen bonds was slightly decreased whereas the protein-Gdm hydrogen bonds were increased with their concentration. These fractions were comparable with that of Arg for both the proteins.

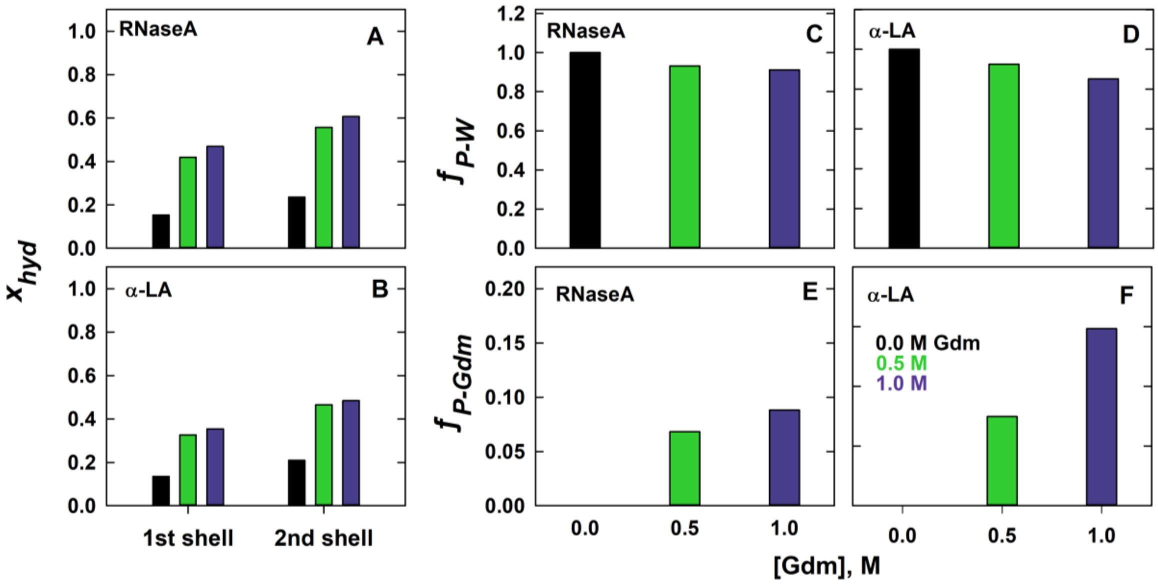

Figure 4.13. Hydration fraction calculated at the first and second hydration shells of around RNase A (A) and α-LA (B). Fractions of H-bonds between protein-water (C & D) and protein-Gdm (E & F) for RNase A and α-LA in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of Gdm.

4.4.4. Residue-level interaction analysis:

The fraction of interactions between individual amino acids on the protein and the added cosolvents were evaluated from their respective MD simulation trajectories. For a better understanding, the total interactions were further divided into four possible types: i) protein backbone (BB) and amino acid backbone (BB) interactions, 2) protein-BB and amino acid-side chain (SC) interactions, 3) protein-SC and amino acid-BB interactions and 4) protein-SC and amino acid-SC interactions (Figures 4.14 and 4.15A&C). In order to identify the residue-specificity in the protein-amino acid interactions, the number of protein-SC and amino acid-SC interactions was calculated for each amino acid in the protein. The other three types of interactions which would be common across the proteins and "non-residue specific" were also evaluated for each amino acid for comparison.

4.4.4.a. Stabilizing amino acids – Lys, Aps, and Glu**:** The fraction of protein-SC with amino acid-BB interactions was relatively higher in all the simulations performed in the presence of stabilizing amino acids (stAAs) for both the proteins (Figure 4.14). It was followed by protein-SC with amino acid-SC interactions in the case of Lys and Glu whereas protein-BB and amino acid-BB in Asp.

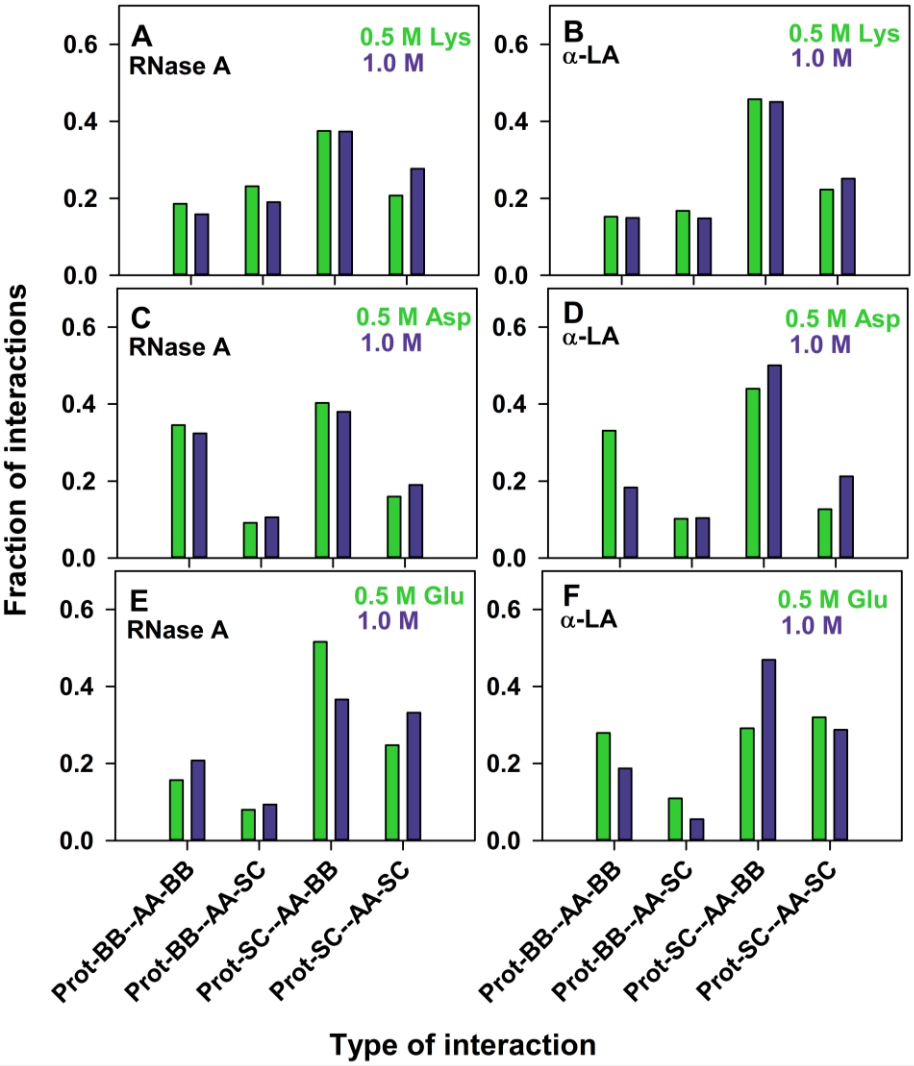

Figure 4.14. The fractions of all the four possible interactions between protein and the stabilizing amino acids (Lys, Asp, and Glu) calculated for RNase A (left panels) and α-LA (right panels). For more details refer to section 4.4.4.

Figure 4.15 shows that lysine preferably interacts with the side chains of positively charged protein residues. At higher concentrations, the contacts were more with glutamine Gln and Asn in both the proteins along with Arg in RNase A and histidine (His) in α-LA. In the cases of Asp and Glu, the SCs of these residues specifically interacted with the basic residues (Arg, Lys and His) along with a few polar residues in both RNase A and α-LA. Almost no interaction was observed with the acidic amino acids. The non-residue specific interactions (Figure 4.16) were mostly found on the charged and polar residues which could be attributed to the solvent exposure of these residues in the globular proteins.

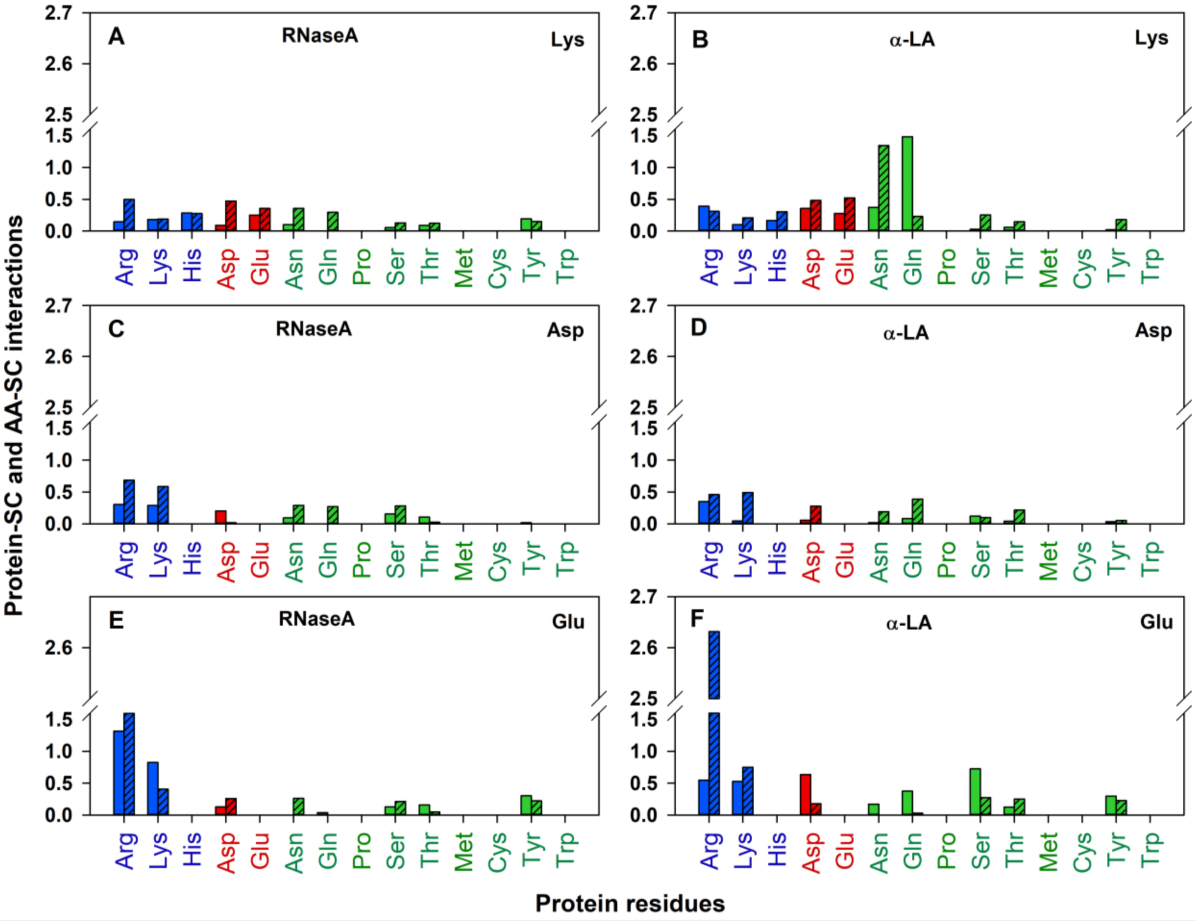

Figure 4.15. The average number of interactions between the protein side chains and the side chains of the added stabilizing amino acids calculated for RNase A (A, C & E), and α-LA (B, D & F). The plain and crossed bars represent the presence of 0.5 M and 1.0 M of stAAs in the simulated system, respectively. The hydrophobic amino acids which do not show any interactions are not presented.

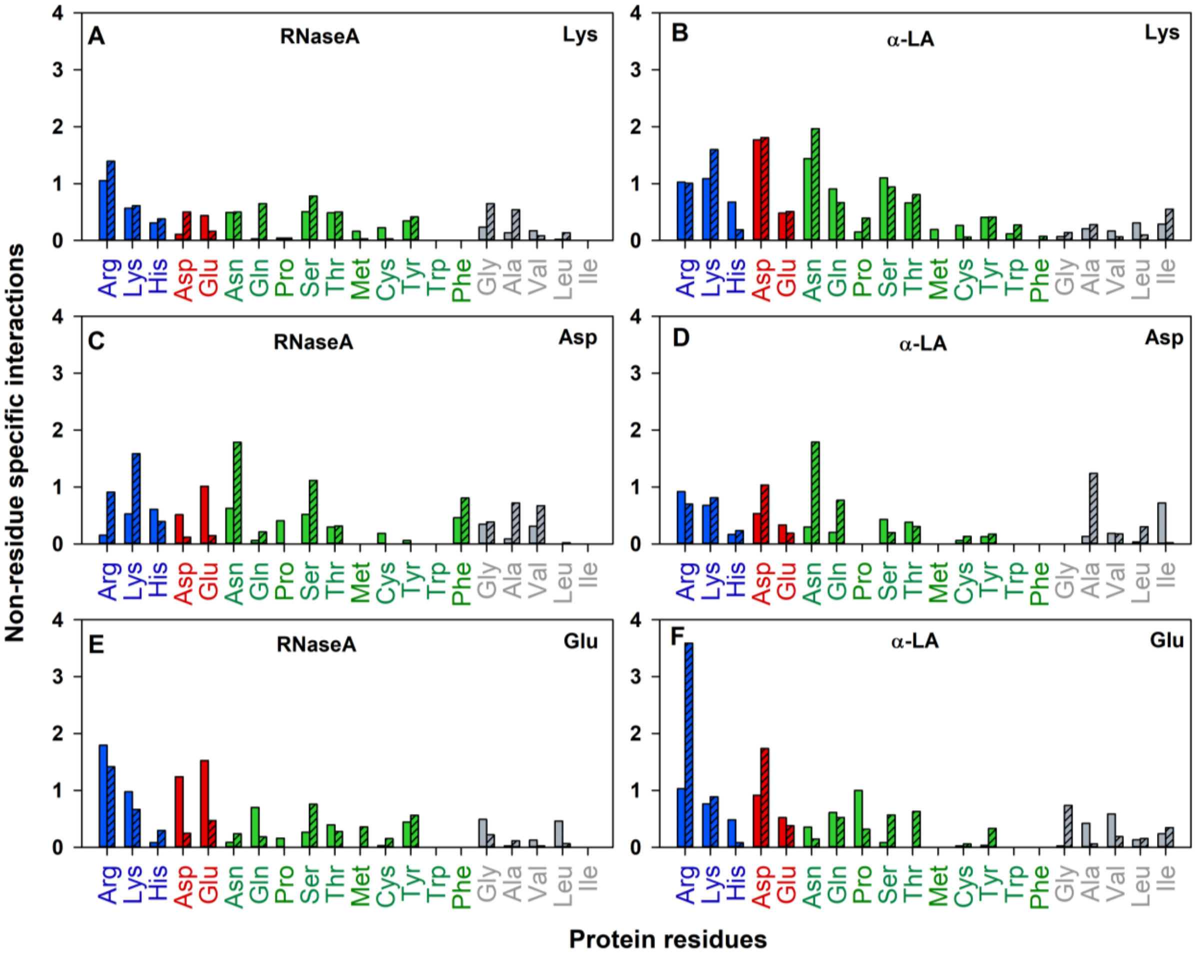

Figure 4.16. The average number of non-residue specific interactions (for details refer the text in section 4.4.4) of added amino acids with RNase A (A, C & E), and α-LA (B, D & F). The plain and crossed bars represent the presence of 0.5 M and 1.0 M of stAAs in the simulated system, respectively.

***4.4.4.b. Destabilizing cosolvents – Arg and Gdm*:** In the case of Arg, the major contributions were from protein-BB with amino acid-SC interactions and Protein-SC with amino acid-SC interactions (Figure 4.17A and C). As Gdm is composed of three amino groups in the resonation with the central carbon, the interaction of any of the amino groups with the protein was considered to be the same. Therefore, the interactions were classified only based on the protein-BB and protein-SC. The results showed that Gdm interacted almost equally with the protein-BB and protein-SC in both the proteins (Figure 4.17B and D).

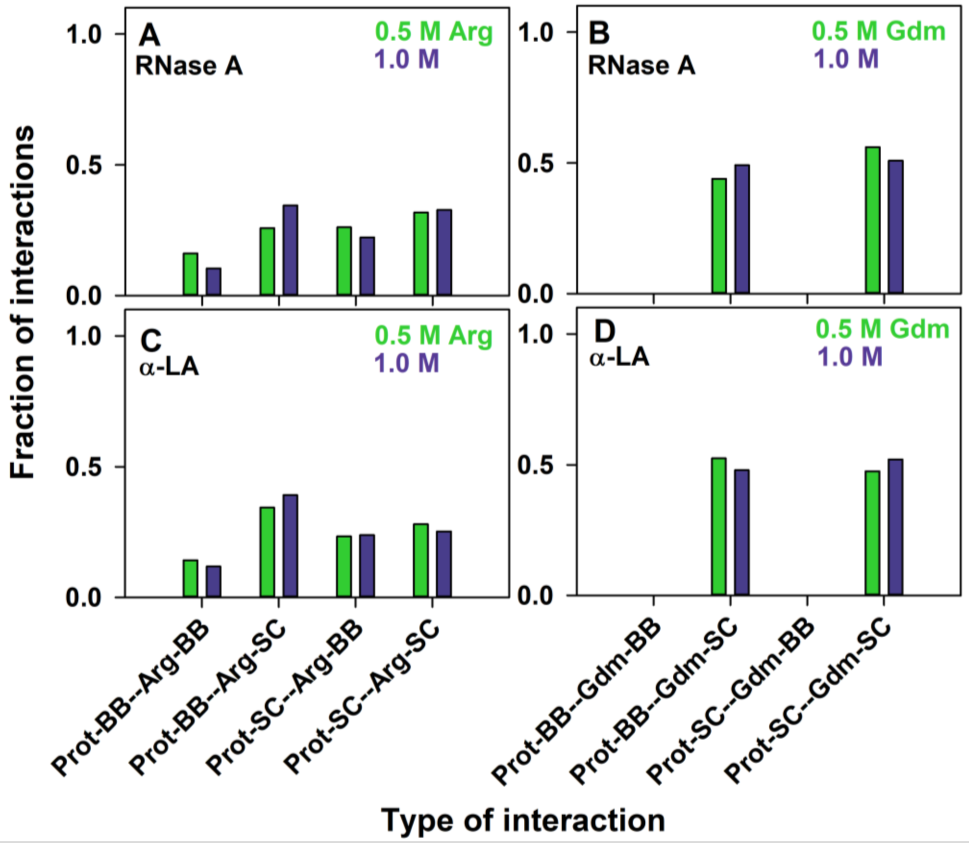

Figure 4.17. The fractions of all the four possible interactions between protein and Arg (left panels), and protein and Gdm (right panels) calculated for RNase A and α-LA.

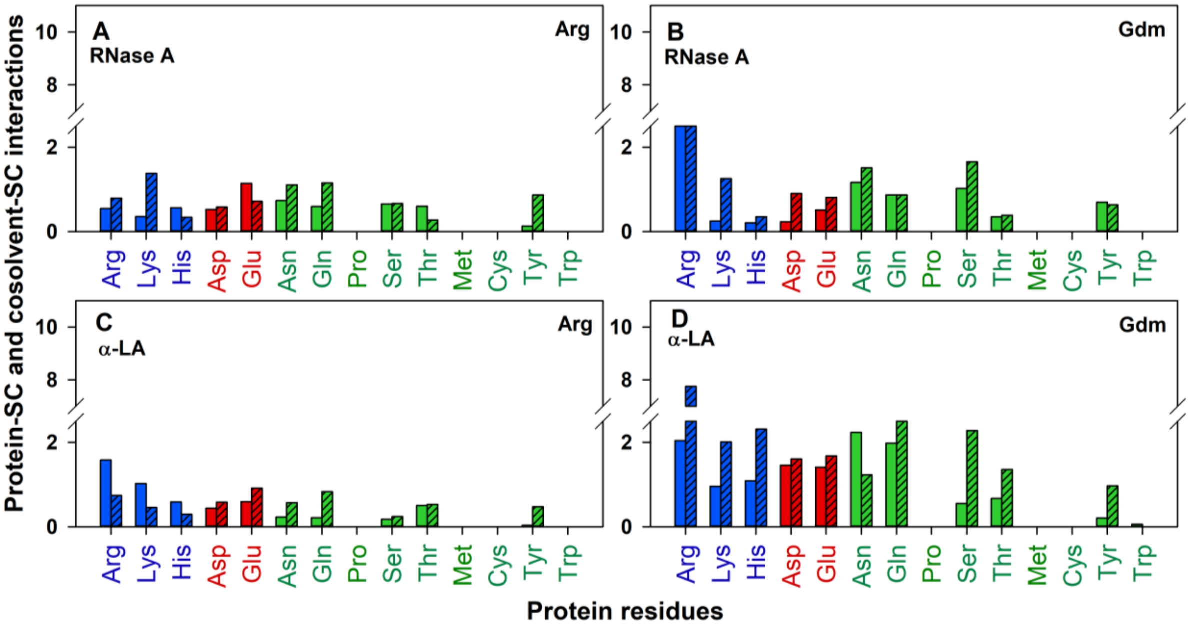

Figure 4.18. The average number of interactions between the residues of the protein and Arg (A and C), and the residues of the protein and Gdm (B and D). The plain and crossed bars represent the presence of 0.5 M and 1.0 M of stAAs in the simulated system, respectively. The hydrophobic amino acids buried in the hydrophobic core which don't show any interactions are not shown.

Further, residue level contacts between the proteins and the cosolvents were also evaluated. Arg side chain interacted mostly with the polar and charged amino acids and the interaction with hydrophobic residues was meagre (Figure 4.18). The non-residue specific interactions were also found to be predominantly with polar and charged residues probably due to their higher solvent exposed surfaces.

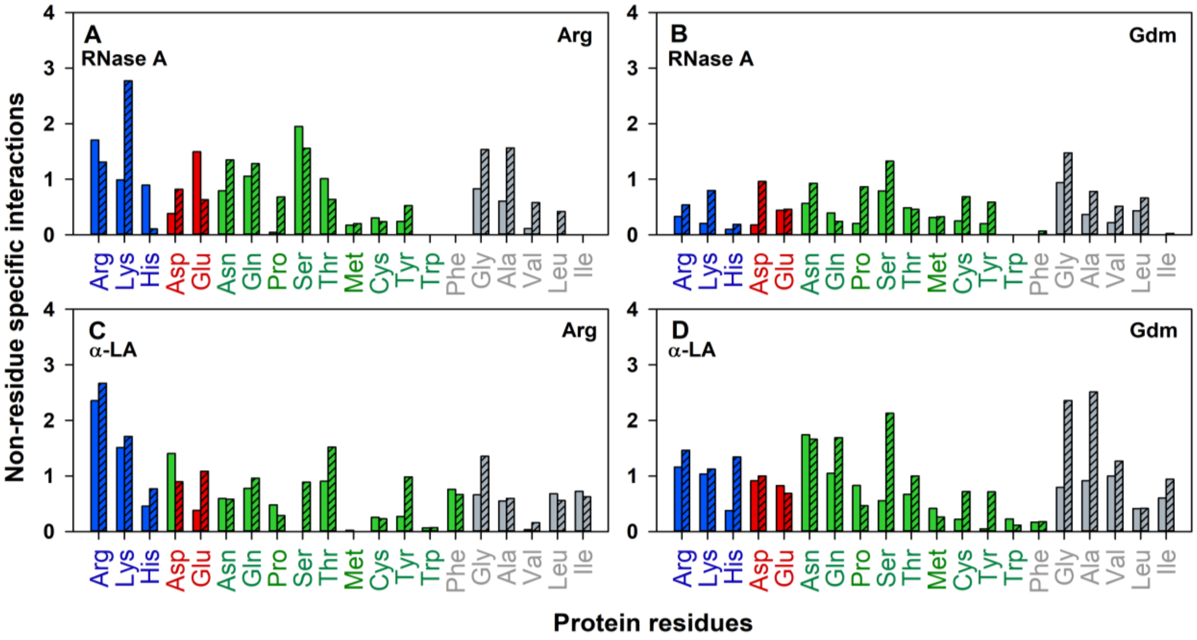

Figure 4.19. The average number of non-residue specific interactions between the residues of RNase A and the stabilizing amino acids (A, C & E), and the residues of α-LA and the stabilizing amino acids (B, D & F). The plain and crossed bars represent the presence of 0.5 M and 1.0 M of stAAs in the simulated system, respectively.

Though Gdm also showed similar interactions, the number of interactions was found to be significantly higher than Arg and even more than the other charged amino acids. Moreover, both Arg and Gdm had significant interactions with the protein-BB atoms across all the amino acids on the surface of the proteins (Figure 4.19). These differences suggest that the direct interaction sites for destabilizing cosolvents (Arg and Gdm) on the protein might be higher than the sites for stAAs.

4.4.5. Energetics of the interactions:

The interaction energies were analysed to understand the driving force for the cosolvent-induced changes on the protein stability. The total interaction energy between the protein and water was slightly decreased for RNase A whereas it was marginally increased for α-LA upon the addition of amino acids or Gdm (Figures 4.20). The analysis of interaction energies between the protein and the added cosolvents revealed that the total energy was increased with the increasing concentration of the cosolvent (Figures 4.21 and 4.22). The energy was higher with the addition of Arg or Gdm compared to other cosolvents.

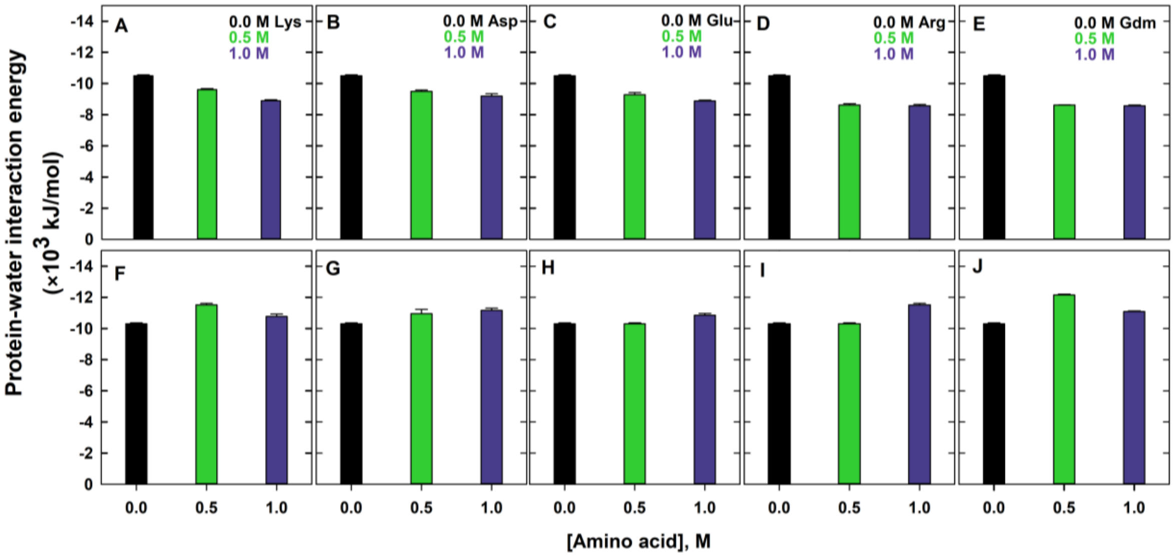

Figure 4.20. Interaction energy between protein and water calculated for RNase (A-E) and α-LA (F-J) in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of the added cosolvent, Lys (A & F), Asp (B & G), Glu (C & H), Arg (D & I) and Gdm (E & J).

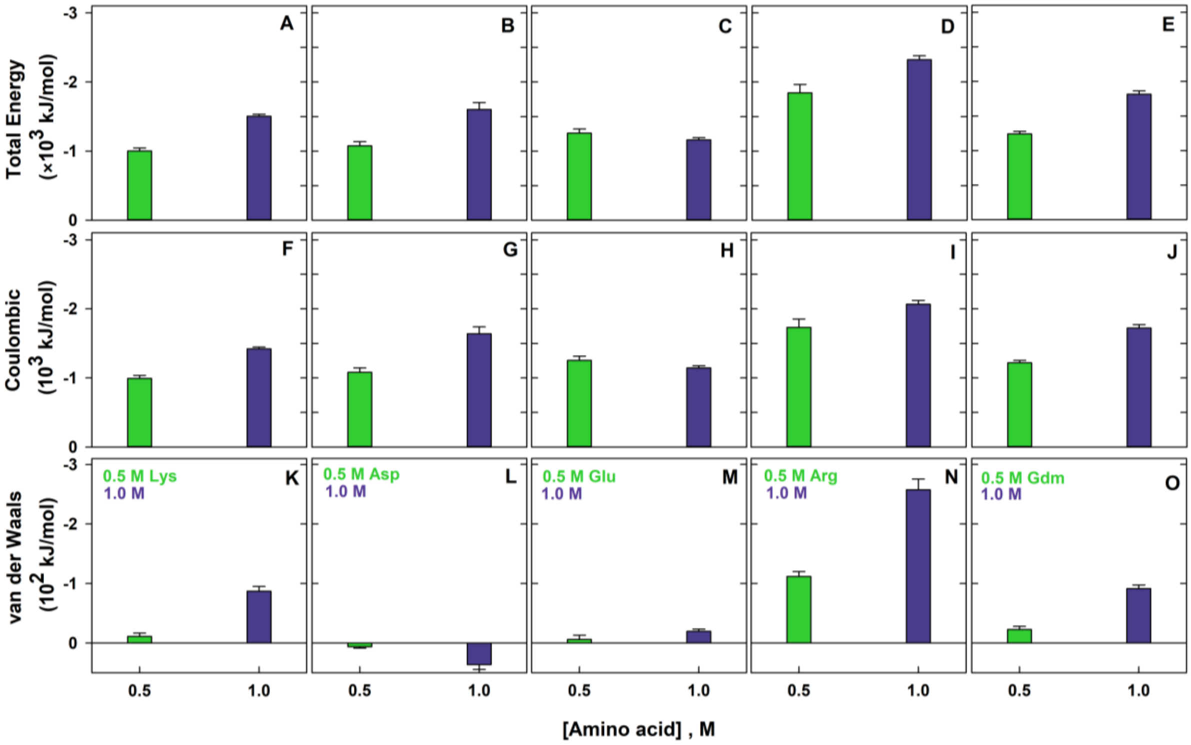

Figure 4.21. The total interaction energy (upper panels) between RNase A and the added cosolvent Lys (A), Asp (B), Glu (C), Arg (D) and Gdm (E) calculated in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of the cosolvent. The total interaction energy is further divided into coulombic (middle panels) and van der Waals interactions (lower panels).

However, in all the cases, the contribution of coulombic energies was predominant (>10 times) than their respective van der Waals contributions in both the proteins. The van der Waals interaction between RNase A and Asp, and α-LA and Gdm were in fact slightly unfavourable.

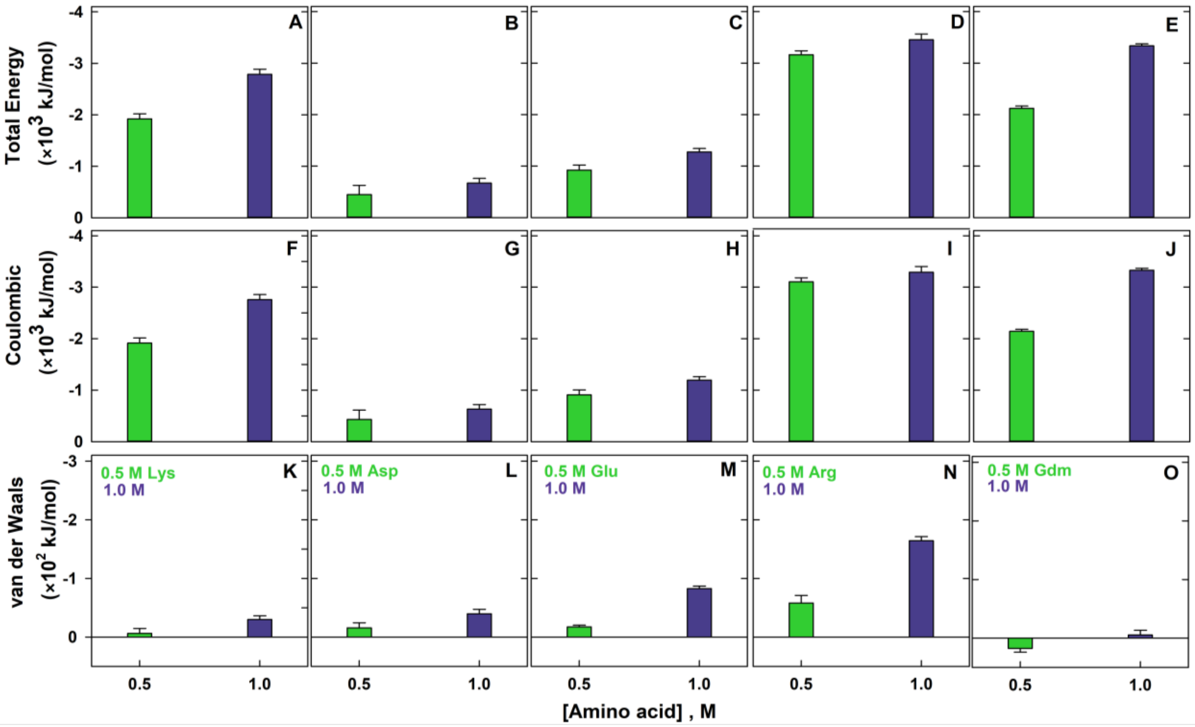

Figure 4.22. The total interaction energy (upper panels) between α-LA and the added cosolvent Lys (A), Asp (B), Glu (C), Arg (D) and Gdm (E) calculated in the absence (black) and in the presence of 0.5 M (green) and 1.0 M (purple) of the cosolvent. The total interaction energy is further divided into coulombic (middle panels) and van der Waals interactions (lower panels).

4.5. Discussion:

Though there are experimental studies on the stabilizing nature of amino acid osmolytes,[69-75] the characterization of the driving forces at the molecular-level is still lacking. All-atom MD simulation is a powerful tool in providing insights into the solvent-induced changes in the structure and dynamics of proteins. Here, molecular dynamics studies of two model proteins in the presence of stabilizing and destabilizing cosolvents are carried out with the objective to analyse the changes in the protein structure, surface solvation, and interaction energetics of the cosolvent at the residue-level.

***4.5.1. Minimal global changes*:**

The RMSD values of the proteins show a sharp increase at the initial simulation time whereas it remains mostly unchanged after 1 ns and the addition of cosolvents (amino acids and Gdm) does not alter the RMSD values. This indicates that no larger conformational changes occurred in both the proteins (Figure 4.1, 4.2A-D and 4.5A-B) which correlate well with the near-UV circular dichroism analysis of both the proteins in the presence of amino acids as discussed in sections 2.4.4 and 3.4.3. In addition, the total SASA values remain almost the same during the course of simulation for both the proteins (Figure 4.1 and 4.2E-H). However, significant differences are noted in the presence of the cosolvents, when the total value is divided into the hydrophobic and hydrophilic surface areas (Figure 4.3, 4.4 and 4.5E-H). The increase in hydrophobic SASA and decrease in the hydrophilic SASA indicates that the cosolvents might have preferably occupied the hydrophilic groups on the protein surface.

***4.5.2. Surface solvation and stabilization*:**

The RDFs calculated for the water distribution around the proteins show that the peak height at 0.28 nm increases with the addition of cosolvents. However, the amino acid distribution decreases around the protein while increasing its concentration (Figure 4.6 and 4.7A-D). Such an increase in the density of water by added cosolvents has been reported earlier in the cases of lysozyme and α-lactalbumin in the presence of proline and Arg respectively.[26, 58] These results propose that the protein surface is preferentially hydrated upon the addition of cosolvents. Further, the variation in the height of the peaks in different cosolvents suggests that the preferential exclusion of Asp and Glu might increase the stability of the proteins compared to other cosolvents. Experimental results also showed that (Section 3.5.1) these amino acids render higher stability to the proteins. A study on the stability of *Drosophila* Su(dx) protein (ww34) in the presence of Arg and Glu illustrated that Glu has a low preferential interaction coefficient.[76] Despite the increase in water density around the protein in the presence of Arg and Gdm, the density of these cosolvents around the protein surface is significantly greater (>0.5) compared to any other studied cosolvent (<0.4). This predicts that Arg and Gdm might form more direct contacts with the proteins, thus can act as destabilizing agents.[58, 76] Moreover, both Arg and Gdm show higher preferential interaction with α-LA compared to RNase A. This could be attributed to the weaker stability of α-LA in these cosolvents as observed in the earlier experiments (Sections 2.4, 2.5 and 3.4.2).

Further analysis by the hydration fraction (Figure 4.9, 4.10 and 4.13) suggest that the fraction of water molecules in the first and second hydration shells tend to increase upon the increasing concentration of the amino acids and Gdm which complement with RDF changes. Nevertheless, the fraction of H-bonds between the protein and water almost remains the same or slightly decreases and the protein-cosolvent H-bonds increases (Figure 4.11 and 4.12). These changes might arise due to the replacement of water molecules bound on the surface by the interaction of the cosolvents. At the same time, the cosolvents might increase the strength of the water-water H-bonds in the first and second hydration shells, thus increasing their number density. A few earlier reports also show similar water replacement mechanism upon the addition of polyol classes of osmolytes.[21, 24] Among the cosolvents, Arg and Gdm show marginally lesser hydration fraction and higher protein-cosolvent H-bonding interactions compared to other amino acids. This reemphasizes the fact that these cosolvents preferably interact with the protein molecules by replacing the surface waters, thus destabilizing the proteins.

4.5.3. Residue-level interactions and driving forces:

***4.5.3.a. Stabilizing amino acids*:** Figure 4.14A and B evidences that the SC of Lys interacts with the side chains of polar and charged residues in the protein and particularly with Asn and Gln. The similar interactions are observed when Lys is added to lysozyme as well.[56] Asp and Glu have major interactions with the side chains of basic amino acids followed by other polar residues (Figure 4.15C-F). To the best of our knowledge, this is the first report on residue-level interaction study on Asp and Glu as cosolvents. The non-residue specific interactions of all the amino acids with the proteins have been found to be on almost all the surface-exposed residues and might be significantly contributing to the observed changes (Figure 4.16). The comparison of fraction of interactions via the side chains and the non-residue specific (Figure 4.23) clearly indicates that most of the amino acids interactions with proteins are not residues specific, thus, showing similar stabilization (or destabilization) effects on the proteins with different surface charge.

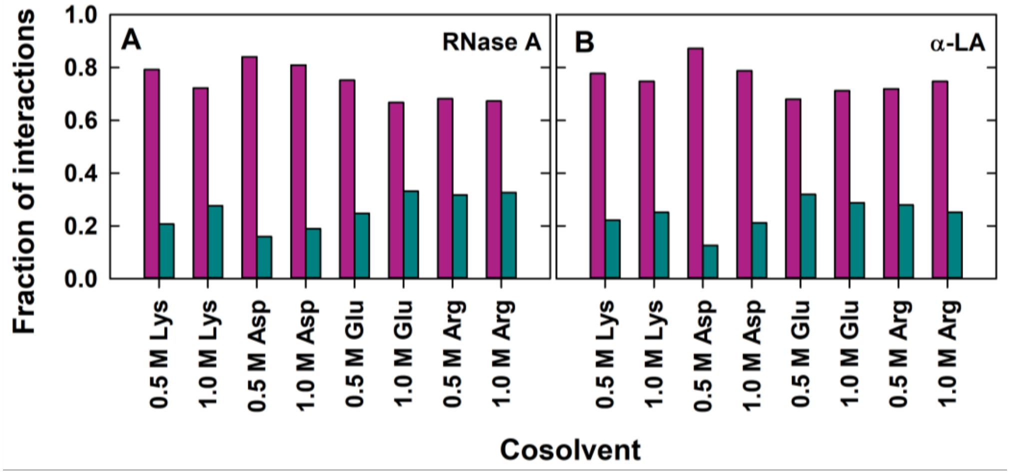

Figure 4.23. The fraction of non-residue specific interactions (pink) and specific side chain –side chain interactions (cyan) between the protein and the cosolvent calculated for RNase A (left panel) and α-LA (right panel) in the presence of 0.5 M and 1.0 M of the amino acids. As Gdm does not have specific side chain, these fractions were not calculated for Gdm interactions.

The interaction energies calculated between protein and water is only slightly altered upon the addition of the stAAs (Figures 4.20). However, the protein-stAA interaction energies are significant and increased with increasing amino acid concentration (Figure 4.21 and 4.22). The coulombic interactions are found to be the major contributing factor in all the amino acids for both the proteins. The less interaction energy of protein with Glu or Asp suggest them as better stabilizing osmolytes with less preferential interactions with the proteins as observed experimentally (Section 3.5.1).

4.5.3.b. Destabilizing cosolvents**:** The number of direct interactions of Gdm and Arg is higher than the stAAs (Figure 4.18 and 4.19) which complements the results observed from the RDFs of respective cosolvents around the proteins. The preferable interacting sites of Arg and Gdm are found to be the SCs of polar and charged residues. Both Arg and Gdm show significant interactions with the backbones of all the residues in the proteins (Figure 4.19). Gdm shows a few interactions with the Trp residues in α-LA (RNase A does not have any Trp residues) which can be a result of cation-π interactions between Trp and guanidinium as noted in the previous reports.[57, 77] Also, Gdm displays significant interactions with aliphatic residues of the proteins which suggests the possibility of hydrophobic interactions of Gdm with aliphatic residues (Figure 4.19) as observed in earlier MD simulation studies on lysozyme.[56] It has been hypothesized that these hydrophobic interactions might help in solubilizing the proteins, thereby resulting in destabilization of the proteins.[78-79] Arg also shows considerable interactions with aliphatic residues which could be attributed to its destabilizing activity similar to Gdm. Further, the presence of Arg and Gdm (Figure 4.21 and 4.22) does not show larger changes in the interaction energies as in the cases of stAAs. However, the direct interaction energies between the protein and Arg or protein-Gdm is at least increase by 1.5 fold compared to the stAAs and mainly through the coulombic interactions. This further confirms the preferential interaction of Arg and Gdm on the surface of the proteins.

***4.6. Summary*:**

Various studies involving proteins and osmolytes performed using experimental and computational methods propose different mechanisms for osmolyte-induced protein stabilization. Though these studies largely discuss the effects of polyols, sugars, and methylamines, the nature of interactions between proteins and amino acid class of osmolytes is not well explored. This chapter discusses the molecular-level interactions of four charged amino acid osmolytes Arg, Lys, Asp, and Glu with two model proteins RNase A and α-LA using MD simulations. The RMSD and SASA values suggest that these amino acids do not induce larger structural changes in the proteins as observed in the spectroscopic studies. RDFs, hydration fractions and H-bond analysis reveal that the distribution of water is increased in the presence of these cosolvents. Further, the extent of increase in water content around the proteins is relatively less in the case of Arg and Gdm whereas the interaction between the protein-cosolvent is higher compared to the other amino acids. Also, the interaction energies indicate that the protein-water interactions energies have only trivial changes whereas the protein-Arg and protein-Gdm interaction energies are high. These energies are mostly due to charge-charge interactions between the protein and these cosolvents. The hydration around the protein increases in the order of Glu > Asp > Lys >Arg > Gdm whereas the reverse order is observed for direct interactions. These results clearly indicate that the combined effects of preferential hydration and direct interaction determine the effect of cosolvents on the proteins. The increase in hydration by the cosolvent or the increase in the direct interaction of the cosolvent stabilizes or destabilized the proteins, respectively. While both Arg and Gdm are found to destabilize the proteins through direct interaction, the effect of the combination of these two non-compatible osmolytes (either counteracting or synergistic) is still a question which has been examined in later chapters.

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Chapter 5

Counteracting Effect of Charged-Amino acids Against the Destabilization of Proteins by Arginine

5.1. Abstract:

Proteins are surrounded by different kinds of osmolytes inside the cells. Understanding the simultaneous effect of the osmolytes, although complicated, is of great importance. The expansion of our knowledge on mixtures of osmolytes will widen their applications. The combinatorial effects with methylamines are majorly found in the literature; however, such studies are limited on the amino acid class of osmolytes. The present study examines the counteracting abilities of the stabilizing amino acids against Arg on RNase A and α-LA. The thermal stabilities of the proteins in the presence of mixture of osmolytes are monitored by the absorption changes in the proteins. The results show that Glu could counteract Arg at the lowest fraction in the mixture. Lys requires nearly equimolar concentration whereas Asp needs almost double the concentration to counteract Arg induced destabilization of the proteins. At higher concentrations, the counteracting ability of Asp and Lys is similar in both the proteins. The counteracting ratio might slightly vary among the proteins and it is not necessary that the amino acid providing higher stability to the protein could more effectively counteract Arg. This could be attributed to the possible change in the extent of preferential hydration of the proteins by stabilizing amino acids in the presence of Arg.

5.2. Introduction:

Although there are wide range of osmolytes available for an organism, the selection of osmolytes to balance the cellular equilibrium depends upon the type of stress it is exposed.[1-2] For instance, marine invertebrates, mainly elasmobranch fishes, are found to accumulate several osmolytes such as methylamines, hypotaurine, betaine etc., to sustain the high salt and urea concentration.[2-5] Also, the concentration of TMAO in deep sea animals is shown to increase with an increase in the depth of the sea to overcome the effects of hydrostatic pressure.[3, 6-7] Despite of their higher stability compared to other organisms, even the mammalian cells (renal-medullary, brain and liver cells) are known to accumulate inositol, sorbitol, taurine, creatine, amino acids and glycerol-phosphocholine (GPC) to counteract high salt conditions.[8-9] These findings led the researchers to pursue the studies on mixture of two or more osmolytes to understand whether their role is additive or synergistic or independent on the thermodynamic stability of the proteins against any stress conditions.

A classic example of such a study is the nephron of mammalian kidney. During the process of concentrating urea and salts for excretion, urea diffuses into the adjacent cells, thereby damaging the intracellular proteins present there. However, the nature has adapted a mechanism for the accumulation of counteracting osmolytes in response to urea diffusion.[10-11] Apart from this, other studies on the counteracting osmolytes show that neither their presence in the cells affects the efficacy of urea nor urea interferes with the counteracting osmolytes ability to protect the proteins.[12-13] While another study involving the glycine betaine (GB) and urea shows the enhanced stabilizing ability of GB in the presence of urea compared to GB alone by decreasing the hydrophobic interactions of urea with the peptide.[14] Equimolar concentrations of monosachharides in the mixture exhibits better stabilization of the proteins than the oligosachharide formed from those monosachharides.[15] Recent study on mycobacterial pyrazinamidase (PZase) in the presence of methanol and glycerol shows that glycerol stabilizes the protein more effectively in the presence of methanol by substituting for the hydrophobic interactions of methanol with the protein and increasing the hydrogen bond formation with the hydration shell waters.[16] Moreover, when osmolytes from different classes are combined and used as cryopreservatives, they enhance the cell viability by exhibiting synergistic or additive effects than they are used alone.[17-19] As cellular milieu consists of many osmolytes, it is very unrealistic to assess the effects of individual osmolytes on protein stability or activity and correlate with the ideal *in vivo* conditions. Therefore, there is a need to understand the effects of osmolyte mixtures on protein stability.

A few attempts have been made to examine the effects of combinatorial addition of the amino acids on different properties of proteins. Addition of equimolar concentration of Arg and Glu is found to be preventing the aggregation and enhancing the solubility up to 8.7 times of structurally different proteins.[20] Further, this mixture could stabilize monoclonal antibodies against aggregation in a pH dependent manner.[21] This synergistic effect of equimolar mixtures of Arg and Glu has been attributed to the increased interactions among the additives over the proteins surface, thereby preventing the protein-protein interactions.[22] Though these studies analyse the effect of combinations of amino acids on protein aggregation, the counteracting effects of amino acids against the destabilization effect of Arg on native proteins have not been well explored from a thermodynamic perspective. In this chapter, we examine the effects of stabilizing amino acids Lys, Asp and Glu against the destabilization effects of Arg on two globular proteins, ribonuclease A (RNase A) and alpha-lactalbumin (α-LA). The results suggest that Glu counteracts the Arg more effectively for both the proteins whereas equimolar concentration of Lys and nearly two-fold of Asp is required to bring the proteins to native-like isostable condition. The similar effects observed on both RNase A and α-LA propose that the counteracting effects might also be due to change in the preferential hydration of the surfaces of the proteins as seen in the case of effects of individual amino acids.[23-24]

5.3. Materials and methods:

5.3.1. Materials:

L-Arginine, L-Lysine**,** L-Aspartate, and L-glutamate, sodium phosphate dibasic and sodium phosphate monobasic were purchased from SRL, India. Bovine pancreatic ribonuclease A (RNase A) and bovine α-lactalbumin (α-LA) were from Sigma.

5.3.2. Thermal stability by absorption spectroscopy:

Thermal denaturation of the proteins was performed in the presence of various mixtures of amino acids with different fractions. The methodology followed is same as described in the section 2.3.2 of chapter 2.

5.4. *Results*:

Thermal denaturation in the presence of mixtures of amino acids:

The model proteins, RNase A and α-LA, were destabilized by Arg whereas stabilized by Asp and Lys. Glu also stabilizes these proteins and forms an equilibrium intermediate during thermal denaturation. In order to analyze the counteracting effect of the stabilizing charged amino acids (stAA) against the destabilization effect of Arg, thermal denaturation studies of RNase A and α-LA were carried out in the presence of different molar ratios of Arg:stAA (stAA = Glu or Lys or Asp) as given in Table 5.1. These molar ratios were considered based on the stabilizing abilities of the stAAs.

Table 5.1: Molar ratios of mixers of amino acids

Arg:Glu	0.5:1	1:1	2:1	3:1	5:1	6:1
Arg:Asp	0.33:1	0.5:1	1:1	2:1	3:1	-
Arg:Lys	0.33:1	0.5:1	1:1	2:1	3:1	4:1

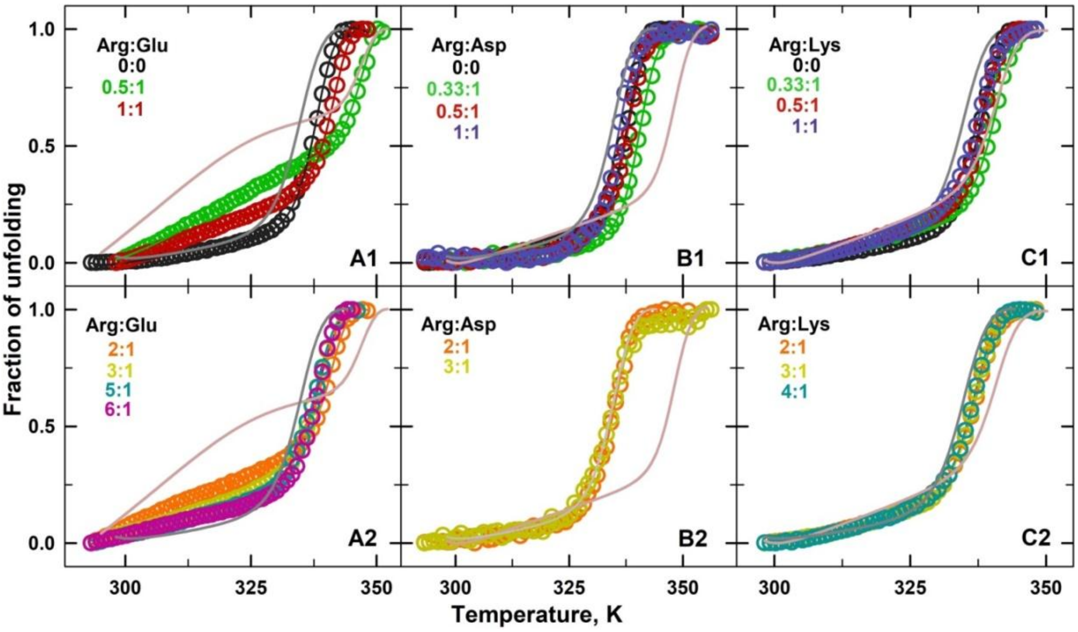

Figure 5.1. Thermal denaturation of RNase A carried out in the presence of different ratios of Arg:Glu (A1 & A2), Arg:Asp (B1 & B2), and Arg:Lys (C1 & C2) are represented by circles. The circle colors correspond to the amino acid ratios as mentioned in the respective figure labels. The solid lines with respective colors on the circles are data fits using equation 2.1 or 2.2. The grey solid lines in all the panels represent the thermal denaturation of RNase A in 1 M Arg and the salmon lines represent in the presence of 1 M of respective stAAs (Glu or Asp or Lys).

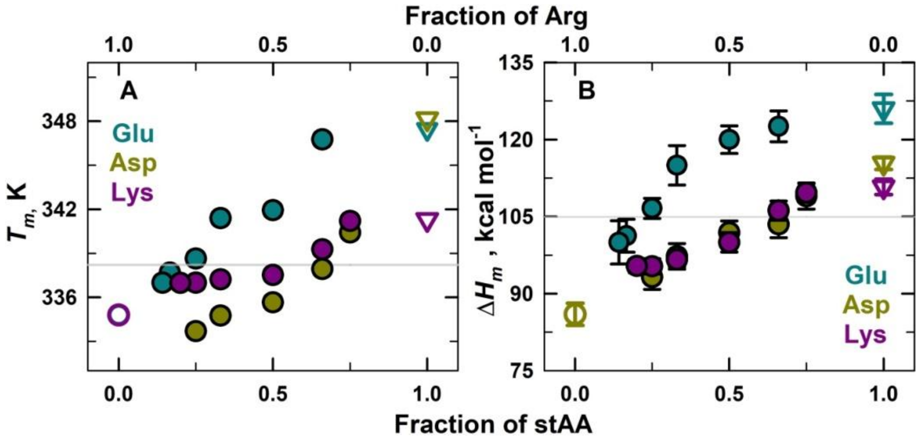

Figure 5.2. (A) Thermal transition midpoint (T_m) and (B) enthalpy of unfolding (ΔH_m) calculated from the thermal denaturation of RNase A in the presence of different ratios of Arg:Glu (cyan), Arg:Asp (green), and Arg:Lys (pink) presented as filled circles. The open circle represents thermal denaturation of RNase A in 1 M Arg and the inverted triangles are in the presence of 1 M of respective stAAs (Glu or Asp or Lys).

In the case of RNase A, the mixure of Arg and stAAs increased the thermal stability compared to the protein in Arg alone (Fig. 5.1). This was evident from the increase in T_m values of the protein as the fraction of any of the stAA was increased (Fig. 5.2 A). Among the stAAs, Glu could counteract the Arg at the lowest fraction of 0.25, and further increase in Glu concentration increases the stability even beyond the stability of native conformation of the protein. Nevertheless, the intermediate induced by Glu during the thermal denaturation of the protein was completely destabilized by Arg even with the half of its concentration compared to Glu. In fact, gunaidinium hydrochloride (Gdm) has also been shown to destabilize the formation of intermediate at the similar fraction of concentration (discussed in section 2.4.2).[24] Though the minimal ratio of Asp against Arg (1:3) showed additional destabiliztion compared to Arg alone, the further increase in the fraction of Asp increased the stability of the protein (Fig. 5.2 A). The destabilizing effect of Arg was counteracted when the fraction of Asp in the solution was 0.66. Almost equimolar concentration of Lys was found to be compensating the destabilization of RNase A by Arg. It may be noted that Asp provided more stabilization to RNase A, but its counteracting effect on Arg was less than Lys and Glu. These observations were complemented with the enthalpy changes (ΔH_m) calculated from the thermal denaturation studies of the protein in the mixure of amino acids (Fig. 5.2B).

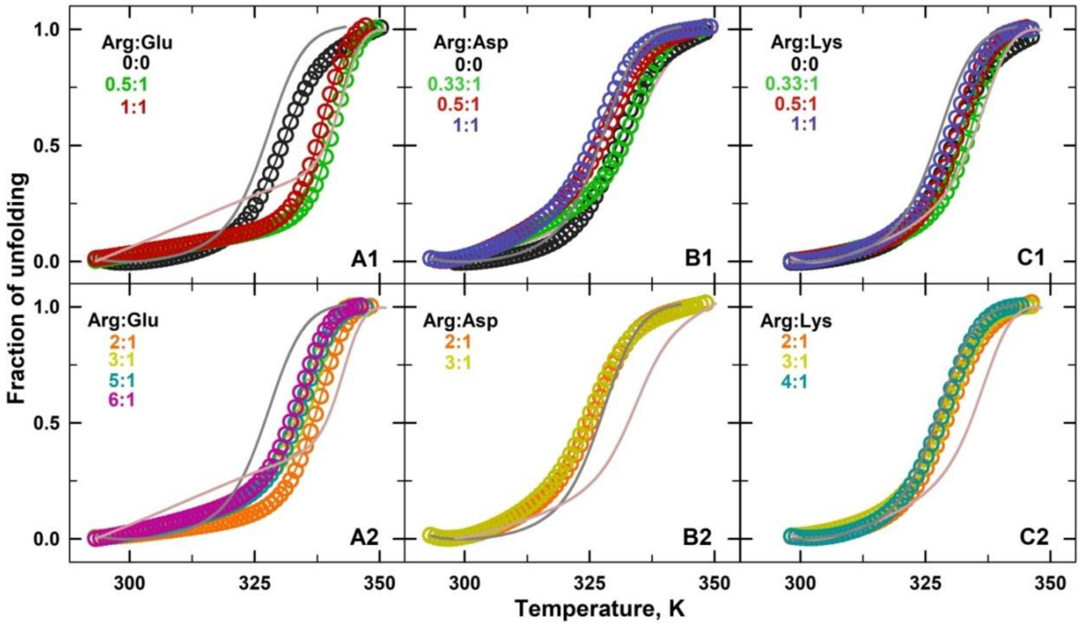

Figure 5.3. Thermal denaturation of α-LA carried out in the presence of different ratios of Arg:Glu (A1 & A2), Arg:Asp (B1 & B2), and Arg:Lys (C1 & C2) are represented by circles. The circle colors correspond to the amino acid ratios as mentioned in the respective figure labels. The solid lines with respective colors on the circles are data fits using equation 2.1 or 2.2. The grey solid lines in all the panels represent the thermal denaturation of α-LA in 1 M Arg and the salmon lines are in the presence of 1 M of respective stAAs (Glu or Asp or Lys).

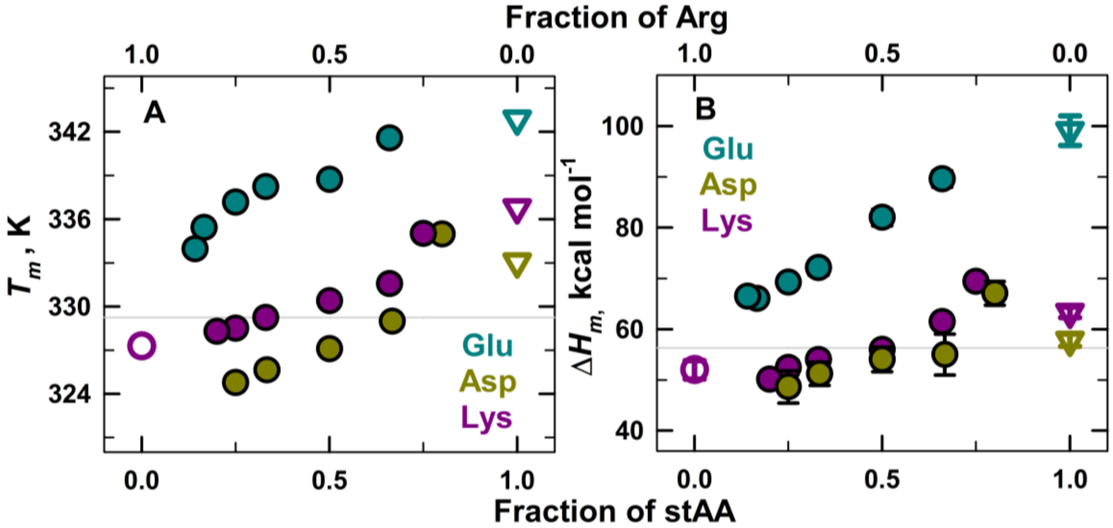

Figure 5.4. (A) Thermal transition midpoint (T_m) and (B) enthalpy of unfolding (ΔH_m) calculated from the thermal denaturation of α-LA in the presence of different ratios of Arg:Glu (cyan), Arg:Asp (green), and Arg:Lys (pink) presented as filled circles. The open circle represents thermal denaturation of α-LA in 1 M Arg and the inverted triangles are in the presence of 1 M of respective stAA (Glu or Asp or Lys).

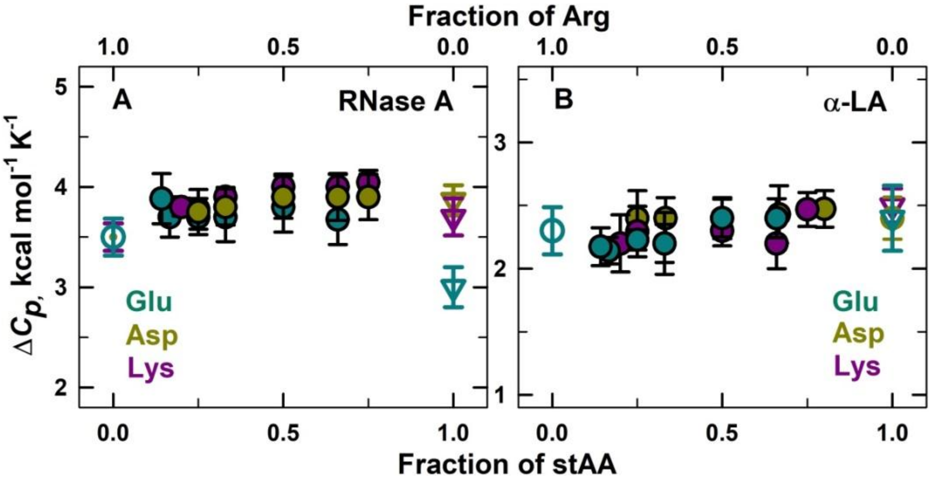

Figure 5.5. The heat capacity changes calculated from the thermal denaturation of (A) RNase A and (B) α-LA in the presence of different ratios of Arg:Glu (cyan), Arg:Asp (green), and Arg:Lys (pink) presented as filled circles. The open circles represent the thermal denaturation of the proteins in 1 M Arg and the inverted triangles are in the presence of 1 M of respective stAA (Glu or Asp or Lys).

There were slight changes observed in the counteracting effects of stAAs in α-LA (Fig. 5.3) compare to RNase A. The T_m and ΔH_m values obtained from the thermal denaturion profiles (Fig. 5.4) clearly showed that even the fraction of 0.14 of Glu (Glu:Arg = 1:6) in the solution was sufficent to counteract Arg. As found in the case of RNase A, Asp at lower fractions showed additional destabilization effect whereas increase in its concentration stabilized the protein and at the fraction of 0.66 it could completely counteract the effect of Arg. Lys showed similar effects on α-LA as observed in RNase A that equimolar concentration of the amino acid could balance the destabilization induced by Arg. The ΔC_p values were not significantly affected during the thermal denaturation of both RNase A and α-LA in the presence of mixtures of amino acids as well (Fig. 5.5).

5.5. Discussion:

As the mixtures of even the same class of osmolytes might not exhibit a simple linear addition of their effects on the stability of proteins, it is essential to investigate their effects in different combinations. The studies focussed on the same chemical class of osmolytes with different charges and also showing difference in their (de)stabilizing effects would provide some basic information on the role of charge-charge interactions in the combinatorial effects of the osmolytes on the proteins. However, partitioning their effect as protein-osmolyte, osmolyte-osmolyte, and water-osmolyte is beyond the scope due to the experimental limitations.

5.5.1. Counteracting effects of stabilizing amino acids:

The addition of Glu and Lys gradually decreases the destabilizing nature of Arg on the proteins. However, at lower concentrations, Asp slightly increases the destabilization effect of Arg and upon further increase in its fraction it counteracts Arg (Figs. 5.2 and 5.4). For further insight, $\Delta\Delta G_T$ values are calculated for the proteins in the presence of mixture of amino acids (Fig. 5.6).[25-26] The extent of resistance provided by stAAs against Arg is not directly correlated to their individual stabilization effects on the proteins. Though Asp provides more stability to RNase A than Lys, the counterbalancing effect of Asp is less than Lys. In case of α-LA, Asp and Lys stabilize the protein to similar extent; however, Lys is more effective in counterbalancing Arg-induced effect than Asp. At higher fractions, both Asp and Lys (0.75) show similar extent of stability in the presence of Arg. For both the proteins, Glu shows the highest stability when it acts alone or while resisting the effect of Arg. This indicates that there might be significant changes in the hydrating ability of the protein surfaces by stAAs in the presence of Arg which determines their counteracting effect. In their combined effect as well, the charged amino acids behave similarly between the proteins irrespective of their surface charges. This reiterates that the water structural rearrangements could be the dominating factor on protein-amino acids interactions.

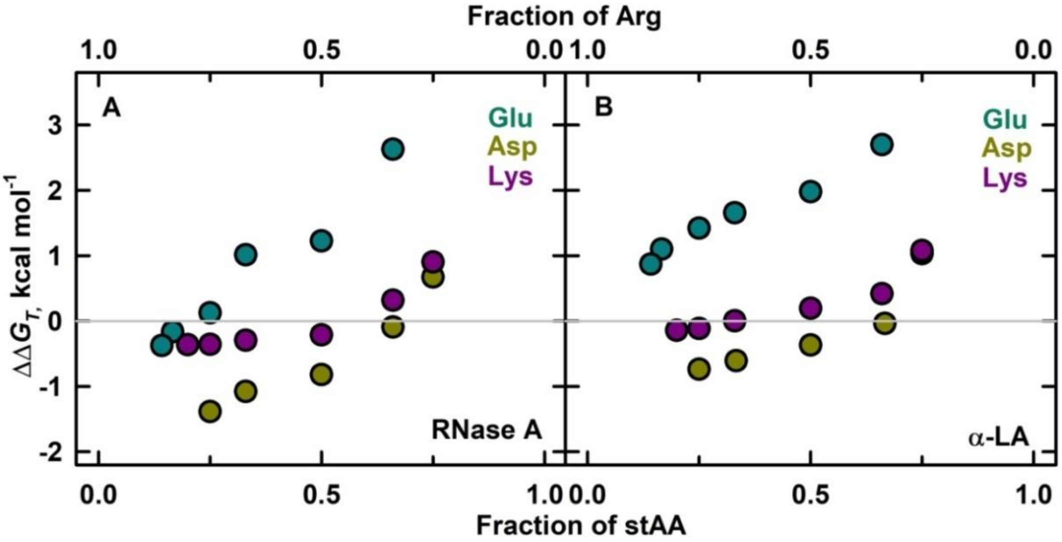

Figure 5.6. Change in the unfolding free energy of (A) RNase A and (B) α-LA measured at their T_m in the presence of different ratios of Arg:Glu (cyan), Arg:Asp (green), and Arg:Lys (pink). The solid grey line represents the $\Delta\Delta G_T$ in the absence of any amino acid which also corresponds to the condition where stabilizing amino acids (stAA) exactly counteract the destabilization of Arg.

5.5.2. Thermodynamic analysis of counterbalancing effect:

To understand the thermodynamic relation between the counteracting effect of stAAs against Arg and their effects of (de)stabilization as individual amino acids, the plots of $\Delta\Delta G_T$ versus individual amino acid concentrations (as discussed in section 3.5) are further analysed for both the proteins. The $\Delta\Delta G_T$ increases with increasing concentration of stAAs whereas decreases for Arg and the values show almost a linear relation against amino acid concentration (Figure 5.7). The linear extrapolation model (LEM) proposed by Greene and Pace is employed to analyse the $\Delta\Delta G_T$ plots.[27-28] The slope of these lines (m_{AA}) represent the extent of stabilization (destabilization in the case of Arg) rendered by per mole of the amino acid.[13, 27-29] The ratio between the slope of stAA and the slope of Arg (m_{stAA} / m_{Arg}) is calculated.[30] This ratio is found to be almost equivalent to the fraction of stabilizing amino acid which could counteract the effect of Arg in the solution for both the proteins. The deviations are noted in the case of Glu which can be attributed to the three-state unfolding of thermal denaturation induced by Glu. Similar studies by Bolen using nine different osmolytes and 46 proteins based on the transfer free energy model also shows deviations in the predicted m-values from the experimental values due to the deviations from two-state unfolding.[29]

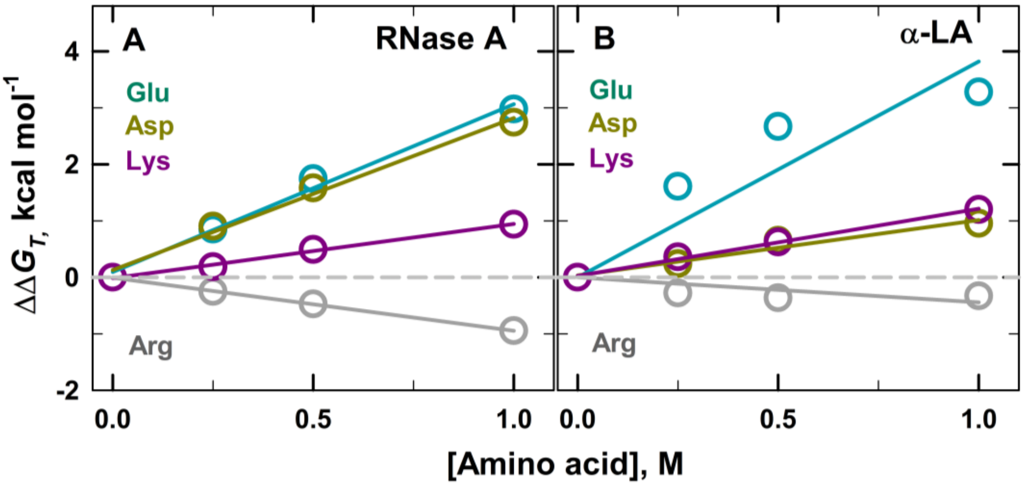

Figure 5.7. Change in the unfolding free energy of (A) RNase A and (B) α-LA measured at their transition midpoint, T_m (i.e., 338 K for RNase A and 329 K for α-LA) upon the addition of different concentrations of Arg (grey), Glu (cyan), Asp (green), or Lys (pink). The solid lines represent a linear fit of the respective data points. The dashed grey line represents the $\Delta\Delta G_T$ in the absence of any amino acid.

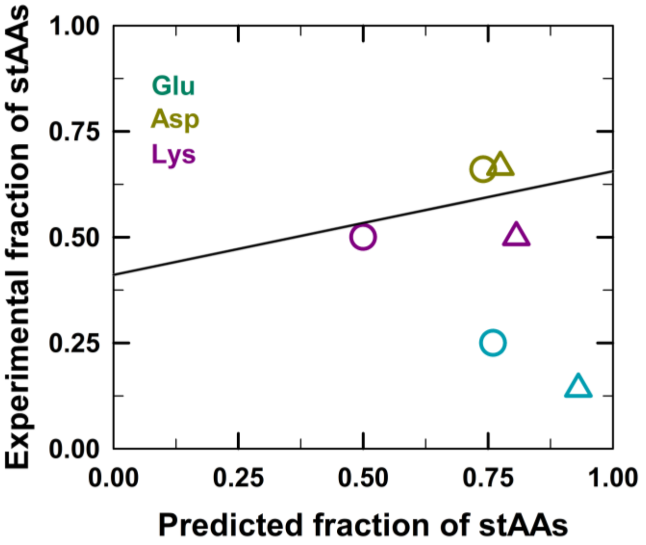

Figure 5.8. Comparison of predicted fractions of stAAs counteracting the Arg with experimentally derived fractions for RNase A (circles) and α-LA (triangles) in the presence of Glu (cyan), Asp (green), or Lys (pink). The solid grey line represents the fit line obtained from linear regression analysis. The data corresponding to Glu was excluded during fitting as it deviates from two-state unfolding mechanism.

The plot of m_{stAA} / m_{Arg} against the experimentally identified counteracting fractions of stAAs against Arg (Figure 5.8) shows a linear relation for both the proteins by excluding Glu. This relation indicates that the decrease in stabilizing free energy of the proteins by Arg could be counteracted by the addition of the fraction of stAAs (Asp and Lys) which would provide the same extent of additional free energy by stabilization.[12 13, 30] Further, the slopes obtained can be used to predict the counteracting fractions (or ratios) of osmolytes at least of the same class, in this study the charged amino acids.

5.5.3. Comparison with other osmolyte mixtures:

Earlier reports have shown that stabilizing osmolytes such as monosaccharides (glucose, fructose, galactose) might exhibit additive effect when used in a mixture whereas the oligosachharides (sucrose, raffinose, stachyose) formed from these monosachharide subunits display a lesser stabilizing effect on the proteins such as RNase A, α-LA and lysozyme.[15, 31] Moreover, the combination of different classes of osmolytes, betaine, sorbitol, TMAO and sarcosine shows synergistic effect on thermodynamic stability of RNase A. However, the mixtures decreased the enzymatic activity of the protein due to the more compact structure induced by the mixture of amino acids than they are used alone. [32] In the case of counteracting effects, the most widely studied combination is the counteracting effect of methylamine against the destabilization of urea. The counterbalancing ratio was earlier proposed to be 2:1 for urea: methylamine[33-35] and the effects of this experimentally obtained molar ratio is equivalent to the sum of the effects of individual cosolvents observed.[33] However, later experiments revealed that this 2:1 counteracting ratio varies with different proteins.[36] In the present study as well the counterbalancing ratio of the destabilizing amino acid by stAAs is varying between the proteins. These results suggest that the combinatorial effects of even the same classes of osmolytes may not be same on different proteins and it might be influence by the mechanism of unfolding such as formation of intermediates. Hence, more studies are required to illustrate the underlying mechanism of combined effects of osmolytes on the stability of proteins.

5.6. ***Summary***:

The counteracting effect of stAAs Asp, Lys, and Glu against Arg on the thermodynamic stability of RNase A and α-LA is examined. Glu is found to be the most effective osmolyte in mitigating the destabilization by Arg. Also, Arg could destabilize the equilibrium intermediate formed by Glu. The fraction of Asp and Lys required to balance the effect of Arg is almost similar in both the proteins. Moreover, except for Glu, the counteracting ratios obtained from the free energy changes-induced ($\Delta\Delta G_T$) by the amino acids are in good agreement with the experimental ratios. This suggests that the counteracting effects are predictable from the free energy changes for the amino acids while the unfolding mechanism of the protein is two-state. As this is found to be common for both the proteins with different charged amino acids, it could also be proposed that the net outcome of the preferential hydration or interaction of the amino acids in the mixtures determine the counteracting effects rather than simple charge-charge interactions between the protein and amino acids.

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Chapter 6

Thermodynamic Stability and Volumetric Analysis of the Effect of Mixture of Denaturants on Globular proteins

6.1. Abstract:

Characterization of structural properties of unfolded states is essential for understanding the protein folding mechanism. Attempts have been made to examine the presence and nature of residual structures in the unfolded states by using mixture of chemical denaturants. As the studies investigating the effects of mixture of denaturants are limited to urea and Gdm, this chapter examines the combined effect of Arg and Gdm, the denaturants possessing similar side chains, on the destabilization of RNase A and α-LA. The thermal stabilities of the proteins in the presence of different molar ratios of the denaturants are monitored by the absorption changes in the proteins. The thermodynamic analysis shows that the mixture of denaturants exhibits synergistic effect on the proteins. Further, the effect of Arg and temperature on the Gdm-induced unfolded states of the proteins is studied by volumetric measurements. The partial molar volume (V^o) and adiabatic compressibility (K_s) of the proteins in the presence of varying molar ratios of Arg-Gdm are evaluated. The decrease in V^o and K_s values with increasing concentration of Gdm suggests that both the proteins might be gradually losing their intra-molecular interactions resulting in decreased internal cavity. The addition of Arg to the Gdm-unfolded states slightly decreased both V^o and K_s values which can be attributed to the increased non-native contacts and the reduction in the hydration of exposed residues upon direct interaction of Arg with the proteins. This suggests that the addition of Arg into Gdm- unfolded state of the proteins might induce a relatively compact state rather than further expansion.

6.2. Introduction:

A comprehensive view of protein folding process involves the understanding of folded, intermediate and unfolded conformations of the proteins and the energy differences among these different states. Decades of research have been dedicated to characterize the folded conformation of polypeptides, their secondary structural propensities, and thermodynamic stability. There are nearly 1.42 lac of experimentally resolved protein structural entries in PDB (Protein Data Bank) repository till date.[1] However, these structures are mostly natively-folded conformations of the proteins and the unfolded structures are less characterized to the atomic-resolution due to their inherent structural heterogeneity.[2-3] As the inter-conversion between different conformational states separated by low energy barriers makes the polypeptide chain more flexible, the experimental determination of the unfolded ensembles become troublesome with X-ray crystallography and NMR methods.[2,4-5] Therefore, the question of whether the unfolded protein has any residual structure remains largely unsolved.[6-7] The little evidences obtained from simulation and multi-dimensional NMR studies on globular proteins suggest that the unfolded states possess some residual structure which may be important for the initiation of folding.[8-12] The unfolded conformational state is denaturant-dependent and their residual structures might consist of non-native interactions.[11,13-14] It is also proposed that the residual structures may serve as nucleation sites for the initiation of protein folding, thus, reducing the conformational sampling space available for folding from fully unfolded states.[11,15] Also, the presence of diverse conformers of unfolded state ensemble suggests the possibility of many folding routes on the folding energy landscape.[4,11]

The radius of gyration (R_g) predicted for a random coil of globular proteins is approximately 43 Å whereas as the R_g for several denatured proteins studied so far is less than this value suggesting the possibility of incompletely unfolded structures.[12] A couple of atomic-level investigations using MD simulation on a hydrophobic polymer and globular proteins have been carried out to examine whether the denatured state can be further expanded to a random coil state by introducing another denaturing cosolvent.[16-17] This was investigated by the addition of urea to the Gdm-denatured state of 32-mer hydrophobic polymer, lysozyme and protein L. The results show co-nonsolvency behaviour by the mixture of urea-Gdm on the polymer[16] and the proteins[17] as well. The co-nonsolvency is the reduction of ‘goodness’ of two solvents (generally chaotropic agents), differencing in their solvent quality, upon their mixing.[18-19] A couple of reasons have been proposed for the chain compaction of the polymers in urea-Gdm mixture: a) the strong electrostatic interaction between the two denaturants enhances the density of urea (urea-cloud) on the polymer surface as Gdm is bound to the polymer, and b) increase in the long-range attractive interactions between the residues of the polymer mediated by the increased urea cloud on the surface. The resulting crowded environment around the polymer surface in the mixture of denaturants induces the formation of non-native contacts leading to the hydrophobic collapse rather than further expansion of the chain. In contradiction to the above, Graziano proposed that it is not the hydrophobic collapse rather it is a compact state.[20] The increased interactions of the denaturants over the protein surface lead to the increased solvent excluded volume and decreased water density in the hydration shell thus leading to the protein compaction.[20] These assumptions are further supported by a single-molecule fluorescence spectroscopy studies on a cold shock protein which also attributes the chain compaction to local-crowding induced by the mixture of denaturant molecules.[21] The limited numbers of available reports have mostly used urea and guanidinium as denaturants to study the unfolded states. To the best of our knowledge, the effect of other classes of denaturants in the presence of either of these denaturants has not been studied.

In order to understand the effects of osmolytes on the different states of the proteins, the preferential interaction and solvation changes of different conformational states have to be quantitatively assessed. However, most of the spectroscopic probes fail to detect the effects of denaturants on the denatured states. The molar volume and compressibility are the fundamental thermodynamic parameters that are sensitive to the solute-solvent interactions.[22] The structural changes observed due to unfolding of the proteins might also be reflected on the volume changes associated with the proteins. The net volume changes associated with the unfolding transition is expected to be the sum of conformational volume (packing volume and void volume) and hydrational volume changes upon denaturation.[22-25] The volumetric measurement of the unfolded polypeptide chains of globular proteins show that partial volume changes upon denaturation is less.[26-27] However, the compressibility is more sensitive to the hydration changes around the protein. The compressibility reduces upon denaturation and attains even a negative value for a completely unfolded protein chain.[26,28] Therefore, measuring the volumetric properties might provide essential information on hydration of protein chains in different conformational states.

As the studies focusing on examining the changes induced by the use of a mixture of denaturants sharing a similar chemical group in their structure are lacking, we examined the effect of the mixture containing Gdm and Arg (a destabilizing osmolyte) on the thermodynamic stability and volumetric properties of RNase A and α-LA. These two denaturants with similar guanidinio group in their structure are chosen to minimize the interactions between them and to examine whether the destabilization effect is additive or reduced or synergistic. Accordingly, the thermodynamic stability of the proteins RNase A and α-LA were examined by following the spectral changes. The density and acoustic measurements were carried out to assess the volumetric changes of the denatured state attained by the addition of Gdm and to evaluate the effect of further perturbation by the addition of another denaturant (Arg) and by increasing temperature. The results show a synergistic effect on the destabilization of both the proteins in the mixture of denaturants except at 2.0 M of Gdm in RNase A. The volumetric results suggest the compaction of unfolded state upon the use of mixture of denaturants.

6.3. Materials and methods:

6.3.1. Materials:

Guanidinium chloride (Gdm), L-arginine (Arg), sodium phosphate dibasic, and sodium phosphate monobasic was purchased from SRL. Bovine pancreatic ribonuclease A (RNase A) and bovine α-lactalbumin (α-LA) were from Sigma. All the chemicals were used without any further purification.

6.3.2. Thermal denaturation studies:

Thermal denaturation of the proteins was performed in the presence of mixtures of varying concentrations of Gdm and Arg. The methodology followed is same as described in the section 2.3.2 of chapter 2.

6.3.3. Volumetric measurements:

Gdm-induced denaturation of proteins was monitored by deducing Partial molar properties from density and acoustic measurements using DSA-5000M (Anton-Paar) instrument on titrating against Gdm up to 5.0 M and 4.0 M for RNase A and α-LA, respectively. The partial molar properties were also measured in the presence of 0.25 M, 0.5 M, and 1.0 M Arg upon Gdm-induced unfolding. All these measurements were acquired at three different temperatures of 293, 303 and 313 K with the help of an in-built temperature controller of the instrument in 20 mM phosphate buffer at pH 7. The partial molar properties were calculated from these measurements as described in section 2.3.6 of chapter 2.

6.4. Results:

6.4.1. Thermal denaturation studies:

The stability of two model proteins, RNase A and α-LA, in the presence of varying molar ratios of Arg and Gdm was studied by the thermal denaturation of the proteins. It was monitored by measuring the changes in their absorbance values. The denaturation profiles of both the proteins followed a simple two-state transition, native ↔ unfolded (Figure 6.1) at all the conditions studied. Upon increasing the concentration of Gdm, in the absence of Arg, the thermal transitions of both the proteins were gradually shifted towards lower temperatures suggesting destabilization of the proteins by Gdm (Figure 6.1A and E). Our earlier results have shown that Arg also destabilizes the proteins (Fig. 3.5.1).

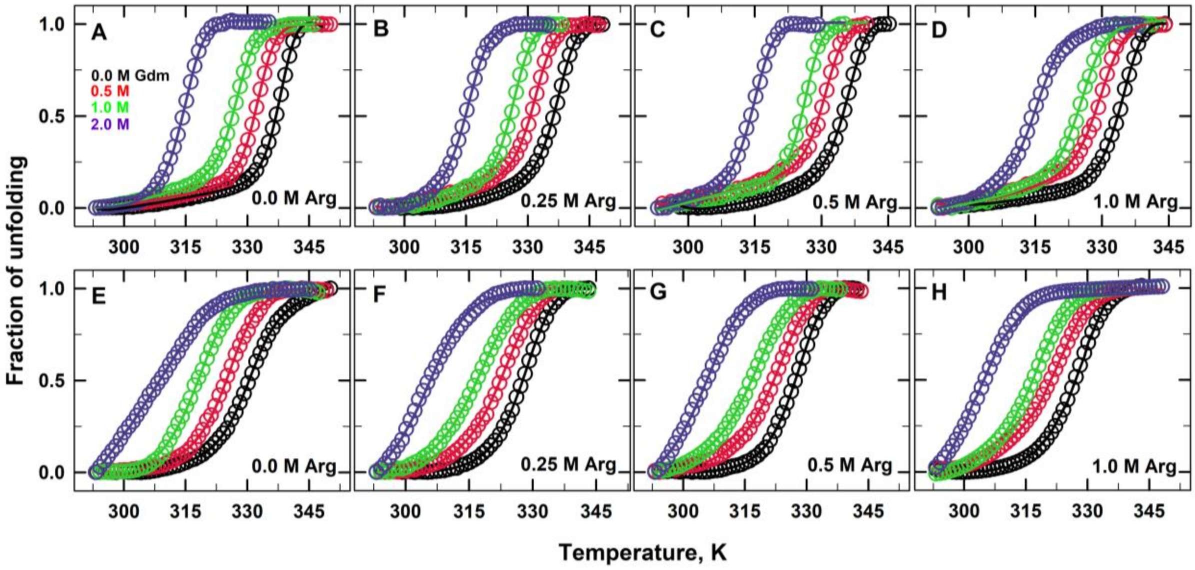

Figure 6.1. The effect of Gdm on the thermal denaturation of RNase A (upper panels) and α-LA (lower panels) in the presence of varying concentrations of Arg monitored using absorbance changes. The color codes represent the concentrations of Gdm as mentioned in the figure label. The solid lines represent the data fitted using equation 2.1.

When the concentration of Gdm was increased, in the presence of 0.25, 0.5, or 1.0 M of Arg, the denaturation profiles were further shifted towards the left indicating the additional destabilization of the proteins in the mixture of denaturants. All the data were fitted to equation 2.1 with two-state assumption (Chpater 2). The thermal denaturation midpoint (T_m) and enthalpy of unfolding (ΔH_m) decreased with the increasing concentration of either of the deanturatns and also in their mixtures (Figure 6.2). The change in heat capacity, ΔC_p (Figure

6.3) in RNase A was trivial in lower concentrations of Gdm (<1 M) whereas at higher concentrations it showed varying effects. α-LA did not show significant changes in ΔC_p values, when the concentration of Gdm was ≤1 M. In 2.0 M Gdm, notable reduction in the ΔC_p values were observed.

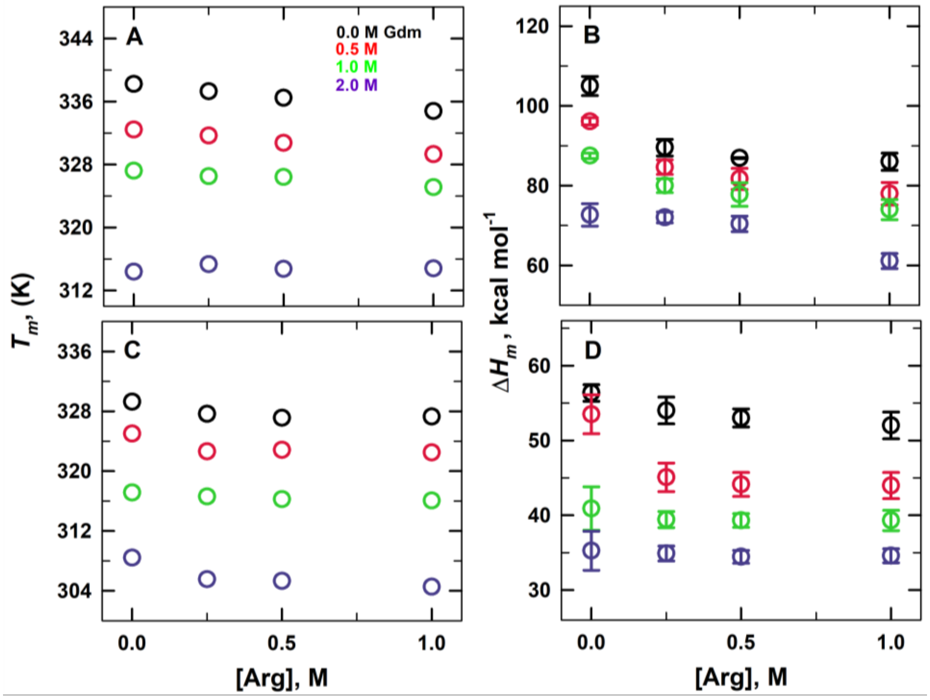

Figure 6.2. Changes in the transition midpoint of thermal denaturation, T_m (A and C) and enthalpy of unfolding, ΔH_m (B and D) of RNase A (upper panels) and α-LA (lower panels) in Arg upon the addition of varying concentrations of Gdm. The color codes represent the concentrations of Gdm as mentioned in the figure labels.

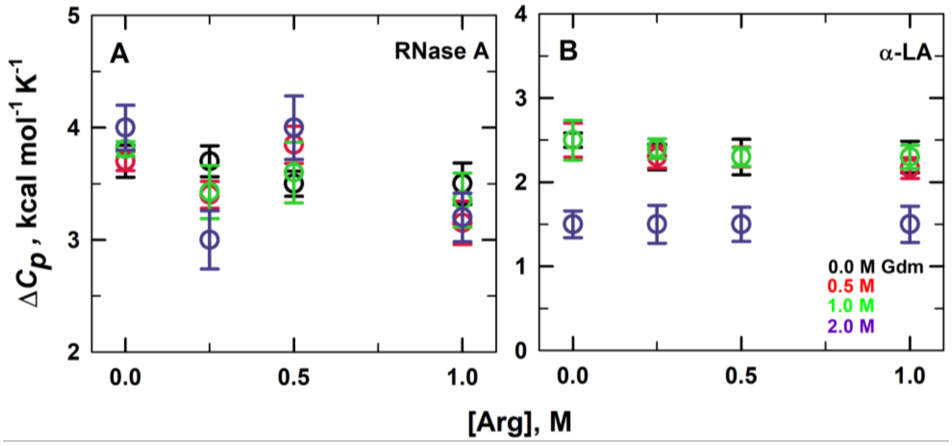

Figure 6.3. The heat capacity change during the unfolding of RNase A (A) and α-LA (B) in Arg upon the addition of varying concentrations of Gdm.

***6.4.2. Volumetric analysis*:**

The partial molar volume and compressibility of the proteins depends on the intrinsic packing and hydrational dynamics upon unfolding. The partial molar volumes (V^o) of both RNase A and α-LA were decreased as the concentration of the Gdm was increased in the solution (Figure 6.4A and E). As the temperature was increased from 293 K to 313 K, the V^o values were slightly increased in all the samples. Similar trends were observed even in the presence of different concentrations of Arg (0.25, 0.5, and 1.0 M) with respect to the increase in Gdm concentration and the temperature as well (Figure 6.4). However, the V^o values were marginally decreased with increasing Arg concentration at all the temperature.

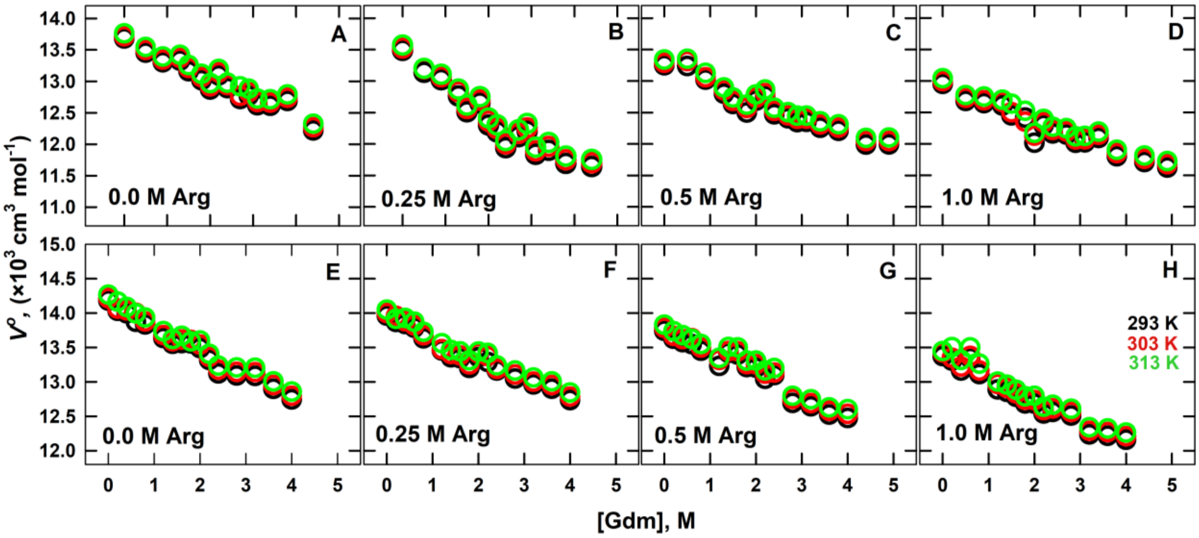

Figure 6.4. Partial molar volumes of RNase A (upper panels) and α-LA (lower panels) evaluated from the density measurements of the proteins in the absence and the presence of Arg with increasing concentrations of Gdm at 293 (black circles), 303 (red circles), and 313 K (green circles).

The adiabatic compressibility (K_s) of the proteins was also decreased with increasing concentration of Gdm in the absence and in the presence of Arg (Figure 6.5). As observed in the case of V^o values, the addition of Arg decreased the compressibility of both the proteins. However, in contrast to V^o values, the K_s values of both the proteins decreased upon increasing the solution temperature and the extent of decrease was gradually reduced at the higher Gdm concentrations.

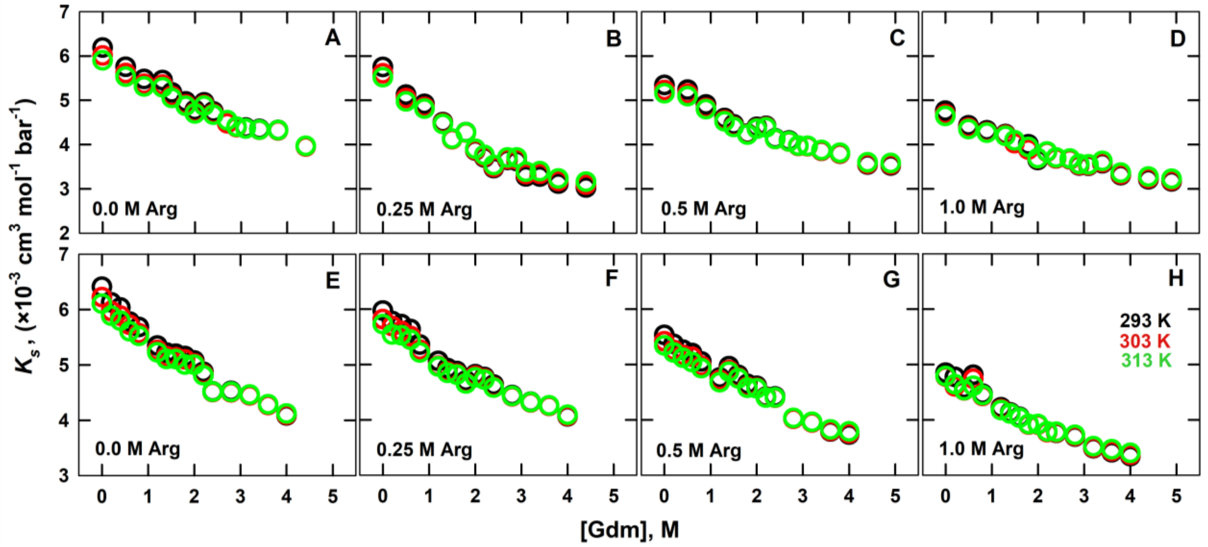

Figure 6.5. Adiabatic compressibilities of RNase A (upper panels) and α-LA (lower panels) evaluated from the density and acoustic measurements of the proteins in the absence and the presence of Arg with increasing concentrations of Gdm at 293 (black circles), 303 (red circles), and 313 K (green circles).

6.5. Discussion:

The dynamics of unfolded chain can be studied by imposing further chemical or physical perturbation on the polypeptide and by monitoring the structural changes and thermodynamic properties. The spectroscopic measurements are generally used to characterize the structural changes along with a few thermodynamic properties such as free energy (ΔG), enthalpy (ΔH_m), entropy (ΔS_m), and change in heat capacity (ΔC_p).[29-32] However, these values cannot directly provide information on the changes in the hydration of the polypeptide chain. Molar volume and compressibility changes evaluated from density and acoustic measurements can quantitatively explain the hydrational changes around the protein chain in different solvent environments.[28, 33-44] The complementary information obtained from these two methods is used here to understand the change in the stability induced by mixture of denaturants and the structural properties of the unfolded polypeptide chains.

6.5.1. Effect of Arg on Gdm-induced destabilization:

The observed decrease in the T_m and ΔH_m of the model proteins (Figure 6.2) in the presence of the mixture of denaturants compared to the values obtained from the individual denaturants suggests the effective destabilization of the proteins by the mixtures. For further quantification of their combined effect, the difference in the unfolding free energy ($\Delta\Delta G_T$) between the native protein and the protein in the presence of denaturants is calculated from their thermal denaturation profiles at the T_m of the respective proteins (Figure 6.6). $\Delta\Delta G_T$ values indicate that the extent of destabilization induced by Gdm is greater than that of Arg. The mixture of denaturants increases the destabilization with an increase in their concentrations except at 2.0 M Gdm in the case of RNase A. However, the destabilization effect of Arg is more at higher Gdm concentration in α-LA.

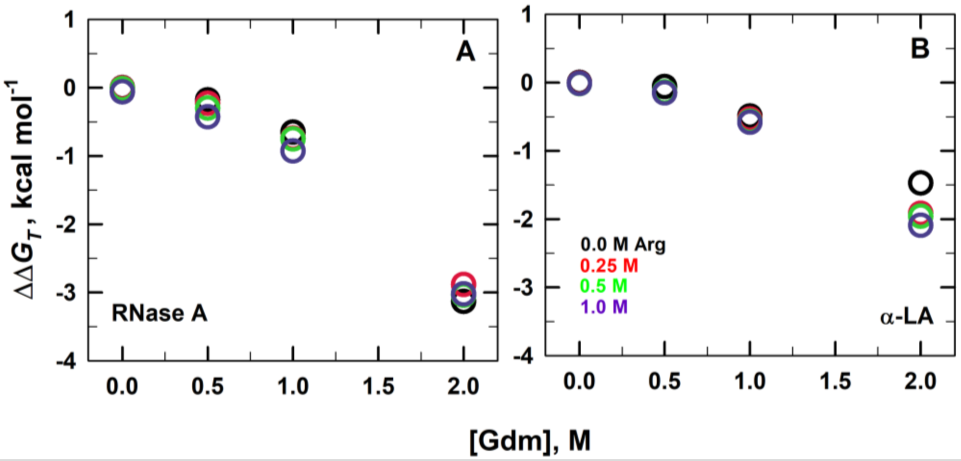

Figure 6.6. The decrease in stabilization free energy of RNase A (A) and α-LA (B) measured at their at T_m (338 K for RNase A and 329 K for α-LA) upon the addition of Arg with increasing concentrations of Gdm as evaluated from the thermal denaturation experiments.

6.5.2. Synergistic effect of denaturant mixtures:

When two osmolytes are mixed in a solution, they may act with simple additive effect[45-47] or non-additive effect[48] or they may synergistically enhance their effect.[48-50] In order to evaluate the combined effect of Arg and Gdm, the $\Delta\Delta G_T$ values of the proteins in the presence of individual amino acids and the mixtures are compared. The values of $\Delta\Delta G_T$ of the mixture of denaturants are more than the sum of $\Delta\Delta G_T$ values of the individual denaturants of corresponding concentrations for both the proteins (Figure 6.7). The only exception is found to be RNase A in 2M Gdm where a marginal counteractive effect between the denaturants is observed. Such synergistic effect has been noted earlier in the studies using mixtures of Arg and Glu or Asp.[51] This effect has been attributed to the interaction between Arg and Glu or Asp which reduces the interaction of Arg with the acidic residues on the protein surface, thus, facilitating the counteraction of Arg’s destabilizing effect by Glu or Asp.[51] Such an increase in inter-solvent interactions which increases the density of cosolvents on the protein surface is even evidenced in simulation studies of Shakhnovich[17] and Trout.[49]

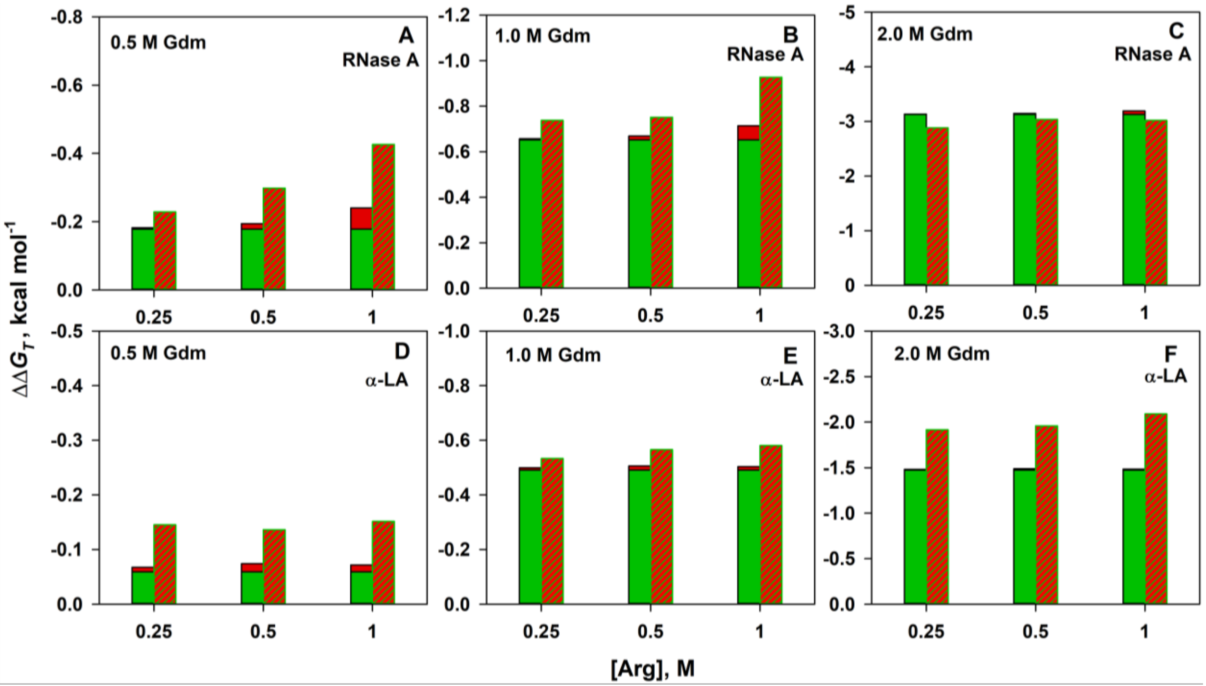

Figure 6.7. Change in the unfolding free energy of RNase A (upper panels) and α-LA (lower panels) measured at their T_m ($\Delta\Delta G_T$). The stacked green and red bars represent the $\Delta\Delta G_T$ values in the presence of Gdm and Arg alone, respectively; The red-green textured bars represent the $\Delta\Delta G_T$ in the mixtures of Gdm and Arg.

6.5.3. Volume and compressibility changes:

In general, it has been suggested that the partial volume changes upon the unfolding of a protein will be less. Though the breaking of intramolecular interactions might increase the partial volume, it might be compensated by the loss of void in the interior of the protein and the hydration of newly exposed polar groups.[26] Kharakoz & Sarvazyan attempted to estimate the volume changes by the addition of hydrational contributions of the surface residues[52] obtained from the partial volume changes of simple amino acids and short peptides.[53] The major limitation of this approach is that the inaccuracy in measuring the solvation changes of partially exposed amino acids.[22, 28] Moreover, such additive relation is less significant in the case of compressibility as K_s values are more sensitive to the hydration and intermolecular (chain-solvent) interactions. K_s values show substantial decrease upon protein unfolding and the completely unfolded protein may even have negative values.[28]

The decrease in the partial molar volume of the proteins with increasing denaturant concentration suggests the gradual loss of internal cavity in the proteins and the hydration of polar groups upon unfolding. It is proposed that the hydrational volume changes on unfolding would be negative for all the globular proteins, thus the total partial volume change associated with unfolding would be negative.[43] Further, the addition of Arg to the native proteins marginally decreases the V^o values even in the absence of Gdm. The near-UV CD spectroscopic study has revealed that the addition of 1 M of Arg does not alter the globular structure of both the proteins significantly (Figure 3.12 and 3.13). Therefore, this marginal decrease could arise from the interaction of surface residues with Arg which reduces the water solvation around the proteins. MD simulation studies suggest that the direct interaction between the protein and Arg is favourable (Section 4.5.3). In addition to this, the V^o values of both the proteins slightly increases with the increase in temperature almost linearly. This might be due to the thermal motion of the exposed residues of the proteins and the average of which may be uniform across the different states of the protein chain.[54-56] An earlier simulation study also shows that the loss of a few intra-molecular contacts in the native state might result in the expansion of interior cavity, thereby increasing the thermal volume.[56] Though thermal volume fluctuations contribute a little to the changes in total volume, its contribution is found to be diminishing upon unfolding as the voids are lost.

The values of partial molar compressibility (K_s) of both the proteins decrease upon increasing the concentration of Gdm (Figure 3.12 and 3.13). This decrease might be due to the cooperative loss of intra-molecular interactions in the protein during unfolding. The decrease in compressibility upon unfolding is in correlation with the previous observations on several other globular proteins.[27, 41, 43] It is also estimated that the hydration contribution of individual amino acids is negative for all the amino acids, as they show greater hydration when surface exposed.[57-59] Further, the increase in temperature might further weaken the intra-chain interactions, thus, the compressibility is reduced. However, as the concentration of Gdm is increased above 2 M (irrespective of the presence or absence of Arg), the compressibility is not altered by change in temperature. Similar results are shown by both the proteins in the presence stabilizing amino acid osmolytes as well (Section 3.5.2). Hence, it is suggested that the thermal motions of surface exposed residues are constrained by the addition of osmolytes. Also, the increase in Arg concentration decreases the K_s values consistently even in the presence of Gdm. As mentioned earlier, Arg alone at 1 M concentration do not significantly unfold the proteins, these reduction in K_s values might arise due to the direct interaction of the osmolyte on the surface residues which weakens the hydration of the protein. It may be noted that V^o and K_s values do not show any additive effects in the mixure of Gdm and Arg as their combined effect on protein surface hydration may be different from their individual effects.

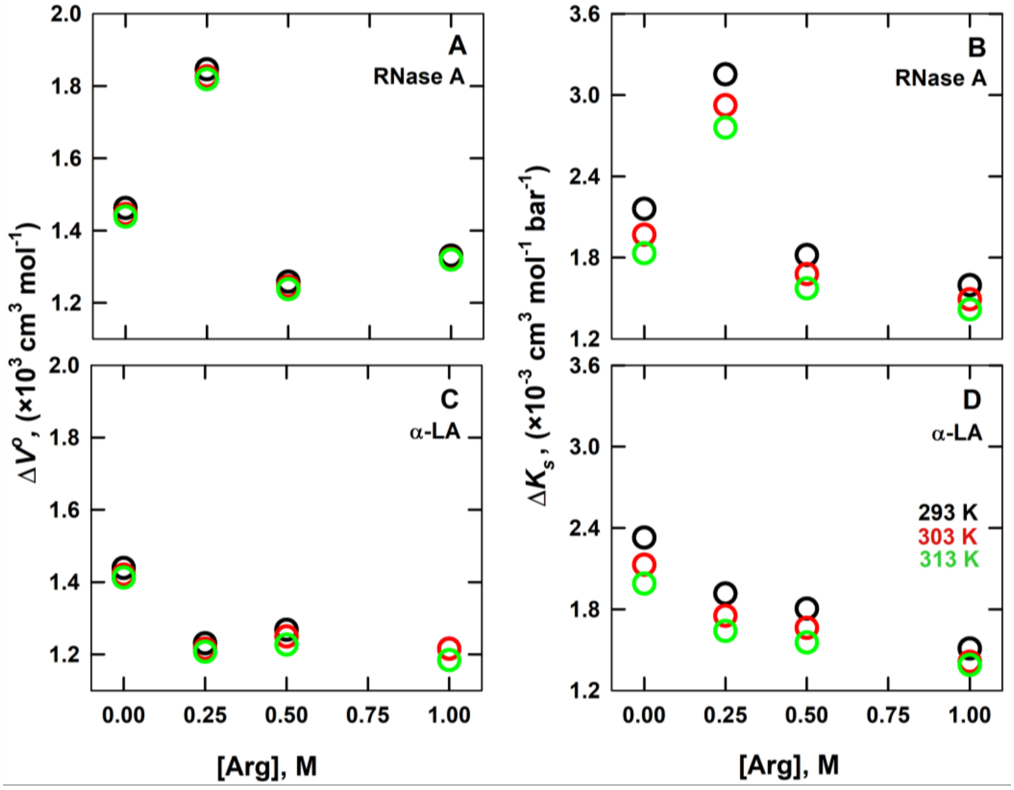

Figure 6.8. The extent of volume (A & C) and compressibility (B & D) changes on the Gdm-induced unfolding of RNase A (upper panels) and α-LA (lower panels) in the absence and the presence of Arg calculated at 293 (black circles), 303 (red circles), and 313 K (green circles).

6.5.4. Unfolded state in the mixture of denaturants:

The extent of change in V^o values (ΔV^o) and K_s values (ΔK_s) during unfolding of the proteins is evaluated at varying concentrations of Arg at different temperatures (Figure 6.8). The decrease in ΔV^o values suggest that the mixture of Gdm and Arg does not show any additive effect on the volume. Considering the fact that Arg does not unfold the native proteins, the decrease in ΔV^o might be due to the formation of additional intra-molecular interactions and decrease in internal cavity of the unfolded chain in the mixture of denaturants. Comparing these results with the synergetic decrease in thermodynamic stability of the proteins indicates that the interactions leading to the compactness of the proteins might be non-native interactions. The non-additive behaviour of ΔK_s values in the mixture of Arg-Gdm also supports the same. The decrease in the extent of reduction in compressibility by the addition of Arg suggests that the proteins attain relatively a compact state rather than further chain expansion upon addition of Arg. These results are similar to the effects observed in the combinatorial effect of Gdm and urea on the proteins.[17, 20] Further, the decrease observed in the ΔV^{o} and ΔK_s upon increasing temperature could be due to the increased thermal motions which might have reduced the compactness of the native state.

6.6. Summary:

The present study discusses the nature of unfolded state of two model proteins in the mixture of denaturants. The spectroscopic, density and acoustic analysis of the proteins provided complementary information on the stability and volumetric changes in the proteins. The Arg-Gdm mixtures synergistically act on the proteins and destabilize the proteins more effectively than they are present alone. The only exception is observed in RNase A at the higher concentrations of Gdm where the destabilization effect of the mixtures of the denaturants is marginally reduced. The partial molar volume and compressibility values of the proteins decrease upon denaturation by Gdm suggesting a gradual loss of interior cavity and native intra-molecular interactions. However, the extent of decrease in both V^{o} and K_s values is reduced and show a non-additive effect in Arg-Gdm mixtures. These results indicate that the unfolded chain of the proteins might attain a compact state with non-native interactions which may increase the interior void and reduce the hydration of polar residues.

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List of Publications

S.No	**Publications**
1	Anumalla, B.; Prabhu, N. P., Glutamate-induced thermal equilibrium intermediate and counteracting effect on chemical denaturation of proteins. *J Phys Chem B* **2018,** *122* (3), 1132-1144.
2	Anumalla, B.; Prabhu, N. P., Counteracting effect of charged amino acids against the destabilization of proteins by arginine. (manuscript under review)
3	Anumalla, B.; Prabhu, N. P., Investigating the protein-osmolyte interactions at the molecular level by molecular dynamic simulations. (manuscript under preparation)
4	Anumalla, B.; Prabhu, N. P., Thermodynamic and volumetric analysis of the effect of mixture of denaturants on globular proteins. (manuscript under preparation)
5	Anumalla, B.; Prabhu, N. P., Charged-amino acids induced effects on the stability and volumetric properties of globular proteins. (manuscript under preparation)

EFFECT OF CHARGED-AMINO ACID OSMOLYTES ON PROTEIN STABILITY: SPECTROSCOPIC, VOLUMETRIC, AND COMPUTATIONAL STUDIES

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